

COMPOSITIONS AND METHODS FOR THE THERAPY, DIAGNOSIS AND MONITORING OF BREAST CANCER

REFERENCE TO RELATED APPLICATIONS

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TECHNICAL FIELD

The present invention relates generally to therapy, diagnosis and monitoring of cancer, such as breast cancer. The invention is more specifically related to specific epitopes of gammaglobulin, to antibodies and immune cells that recognize such epitopes and
10 to methods for detecting gammaglobulin in patient serum. Such peptides, antibodies and cells may be used in vaccines and pharmaceutical compositions for prevention and treatment of breast cancer, and for the diagnosis and monitoring of breast cancers.

BACKGROUND OF THE INVENTION

Breast cancer is a significant health problem for women in the United States
15 and throughout the world. Although advances have been made in detection and treatment of the disease, breast cancer remains the second leading cause of cancer-related deaths in women, affecting more than 180,000 women in the United States each year. For women in North America, the life-time odds of getting breast cancer are now one in eight.

No vaccine or other universally successful method for the prevention or
20 treatment of breast cancer is currently available. Management of the disease currently relies on a combination of early diagnosis (through routine breast screening procedures) and aggressive treatment, which may include one or more of a variety of treatments such as surgery, radiotherapy, chemotherapy and hormone therapy. The course of treatment for a particular breast cancer is often selected based on a variety of prognostic parameters,
25 including an analysis of specific tumor markers. *See, e.g.,* Porter-Jordan and Lippman, *Breast Cancer* 8:73-100, 1994. However, the use of established markers often leads to a result that is difficult to interpret, and the high mortality observed in breast cancer patients

indicates that improvements are needed in the treatment, diagnosis and prevention of the disease.

In spite of considerable research into therapies and diagnostic methods, there is a need in the art for improved methods for detecting and treating breast cancers.

5 The present invention fulfills these needs and further provides other related advantages.

SUMMARY OF THE INVENTION

Briefly stated, the present invention provides compositions and methods for the diagnosis, therapy and monitoring of breast cancer. In one aspect, the present invention provides polypeptides comprising at least 7, preferably at least 9 and more preferably at 10 least 15 consecutive amino acid residues of an epitope of human gammaglobin, wherein the epitope is selected from the group consisting of IDELKECFLNQTDETLSNVE (Pro2; SEQ ID NO: 1); TTNAIDELKECFLNQ (Pro2-3; SEQ ID NO: 2); SQHCYAGSGCPLLENVISKTI (Pro5; SEQ ID NO: 3) EYKELLQEFIDDNATTNAID (peptide 5A; SEQ ID NO: 4) and KLLMVLMLA (mgb 1; SEQ ID NO: 5), such that the 15 polypeptides contain no more than 30 consecutive amino acid residues present within human gammaglobin.

Within other aspects, the present invention provides pharmaceutical compositions comprising a polypeptide as described above and a physiologically acceptable carrier.

20 Within a related aspect of the present invention, vaccines are provided. Such vaccines comprise a polypeptide described above and an immunostimulant.

Within further aspects, the present invention provides antibodies, such as monoclonal antibodies, or antigen-binding fragments thereof, that bind to a gammaglobin epitope as described above, as well as diagnostic kits comprising such antibodies.

25 Also provided are isolated antibodies, or antigen-binding fragments thereof, that specifically bind to glycosylated gammaglobin, and diagnostic kits comprising such antibodies.

The present invention further provides pharmaceutical compositions that comprise: (a) an antibody or antigen-binding fragment thereof that specifically binds to an epitope as described above; and (b) a physiologically acceptable carrier.

Within further aspects, the present invention provides methods for inhibiting 5 the development of breast cancer in a patient, comprising administering to a patient a pharmaceutical composition or vaccine as recited above.

Within further aspects, the present provides methods for determining the presence or absence of breast cancer in a patient, comprising (a) contacting a biological sample obtained from a patient with an antibody or antigen-binding fragment thereof that 10 specifically binds to an epitope as described above; (b) detecting in the sample an amount of polypeptide that binds to the antibody or fragment thereof; and (c) comparing the amount of polypeptide with a predetermined cut-off value. Within preferred embodiments, the antibody is a monoclonal antibody. Step (b) may comprise, for example, a two-antibody sandwich assay.

15 The present invention also provides, within other aspects, methods for monitoring the progression of breast cancer in a patient. Such methods comprise the steps of: (a) contacting a biological sample obtained from a patient at a first point in time with an antibody or antigen-binding fragment thereof that specifically binds to an epitope as described above; (b) detecting in the sample an amount of polypeptide that binds to the 20 antibody or fragment thereof; (c) repeating steps (a) and (b) using a biological sample obtained from the patient at a subsequent point in time; and (d) comparing the amount of polypeptide detected in step (c) with the amount detected in step (b).

Within further aspects, the present invention provides methods for removing tumor cells from a biological sample, comprising contacting a biological sample with T 25 cells that specifically react with a mammaglobin epitope selected from the group consisting of EYKELLQEFIDDNATTNAID (peptide 5A; SEQ ID NO: 4) and KLLMVLMLA (mgb 1; SEQ ID NO: 5), wherein the step of contacting is performed under conditions and for a time sufficient to permit the removal of cells expressing mammaglobin or a peptide epitope

thereof from the sample. Biological samples include, for example, blood and fractions thereof.

Methods are further provided, within other aspects, for inhibiting the development of breast cancer in a patient, comprising administering to a patient a
5 biological sample treated as described above.

The present invention also provides, within further aspects, methods for stimulating and/or expanding T cells specific for mammaglobin, comprising contacting T cells with a peptide comprising the sequence EYKELLQEFIDDNATTNAID (peptide 5A; SEQ ID NO: 4) or KLLMVLMLA (mgb 1; SEQ ID NO: 5), wherein the peptide comprises
10 at least 7, preferably at least 9 and more preferably at least 15 consecutive amino acid residues of human mammaglobin, wherein the peptide comprises no more than 30 consecutive amino acid residues of human mammaglobin, and wherein the contact is performed under conditions and for a time sufficient to permit stimulation and/or expansion of T cells.

15 In related aspects, isolated T cell populations are provided, comprising T cells prepared as described above.

Methods are also provided, within further aspects, for inhibiting the development of breast cancer in a patient, comprising administering to a patient an effective amount of a T cell population as described above.

20 Within further aspects, methods are provided for inhibiting the development of breast cancer in a patient, comprising the steps of: (a) incubating CD4⁺ and/or CD8⁺ T cells isolated from a patient with a peptide comprising at least 7, preferably at least 9 and more preferably at least 15 consecutive amino acid residues of human mammaglobin, wherein the peptide comprises no more than 30 consecutive amino acid residues of human
25 mammaglobin, and wherein the peptide comprises the sequence EYKELLQEFIDDNATTNAID (peptide 5A; SEQ ID NO: 4) or KLLMVLMLA (mgb 1; SEQ ID NO: 5), such that T cells proliferate; and (b) administering to the patient an effective amount of the proliferated T cells.

Methods are further provided for inhibiting the development of breast cancer in a patient, comprising the steps of: (a) incubating CD4⁺ and/or CD8⁺ T cells isolated from a patient with a peptide comprising at least 7, preferably at least 9 and more preferably at least 15 consecutive amino acid residues of human gammaglobin, wherein 5 the peptide comprises no more than 30 consecutive amino acid residues of human gammaglobin, and wherein the peptide comprises the sequence EYKELLQEFIDDNATTNAID (peptide 5A; SEQ ID NO: 4) or KLLMVLMLA (mgb 1; SEQ ID NO: 5), such that T cells proliferate; (b) cloning at least one proliferated cell; and (c) administering to the patient an effective amount of the cloned T cells.

10 Still further methods are provided wherein the presence or absence of breast cancer may be determined in a patient by detecting the level of gammaglobin mRNA in sample of whole blood, or a fraction thereof, obtained from the patient, wherein epithelial cells have been removed from the sample. For example, such detection may be achieved by (a) contacting a biological sample obtained from a patient with an oligonucleotide that 15 hybridizes to a polynucleotide encoding gammaglobin or a complement thereof; (b) detecting in the sample an amount of a polynucleotide that hybridizes to the oligonucleotide; and (c) comparing the amount of polynucleotide that hybridizes to the oligonucleotide to a predetermined cut-off value, and therefrom determining the presence or absence of breast cancer in the patient.

20 Within other aspects, the progression of breast cancer may be monitored in a patient by detecting the level of gammaglobin mRNA in sample of whole blood, or a fraction thereof, obtained from the patient, wherein epithelial cells have been removed from the sample at two different times. For example, such monitoring may be achieved by: (a) contacting a sample obtained from a patient with an oligonucleotide that hybridizes to a 25 gammaglobin polynucleotide; (b) detecting in the sample an amount of a polynucleotide that hybridizes to the oligonucleotide; (c) repeating steps (a) and (b) using a sample obtained from the patient at a subsequent point in time; and (d) comparing the amount of polynucleotide detected in step (c) to the amount detected in step (b) and therefrom monitoring the progression of the cancer in the patient.

These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

5 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a summary of representative rabbit and mouse monoclonal antibodies raised against the human gammaglobin protein. Included is a summary of assays in which these anti-gammaglobin monoclonal antibodies have been used to detect gammaglobin. The epitope binding sequence for each monoclonal antibody (SEQ ID Nos: 11-18) is also listed. Abbreviations are: n.d. = not determined; FACS = fluorescence activated cell sorter; IHC = immunohistochemistry.

Figures 1B-1C present the CDR sequence for rabbit monoclonal antibodies 6A1 (SEQ ID NO: 19), 16D8 (SEQ ID Nos: 20-21), 6B12 (SEQ ID NO: 22), 2D3 (SEQ ID NO: 23), 14A12 (SEQ ID NO: 24), 29C11 (SEQ ID NO: 25) and 31A5 (SEQ ID NO: 26).

15 Figure 2 presents the human gammaglobin amino acid sequence (SEQ ID NO: 27), along with peptide and recombinant regions used for epitope mapping studies. Various peptides (Pro1-9 (SEQ ID NO: 27), Pro-20 (SEQ ID NO: 27) and Glob-2 (SEQ ID NO: 27)) spanning gammaglobin protein sequence were synthesized and used for epitope mapping of the monoclonal antibodies using the ELISA method. Each peptide sequence is 20 indicated in bold and underlined. In addition, an N-terminal recombinant fragment of gammaglobin (SEQ ID NO: 28) was also used for epitope mapping studies.

Figures 3A-3D present epitope mapping data for the rabbit and mouse monoclonal antibodies obtained by the ELISA method. Figure 3A shows the epitope binding regions of the mouse monoclonal antibodies. Shaded areas are considered positive 25 for the antibody. Epitope binding specificity for the affinity-purified rabbit polyclonal 967 is also demonstrated. Figures 3B-3D present epitope mapping data for the rabbit monoclonal antibodies 6B12 (Figure 3B), 29C11 (Figure 3C) and 2D3 (Figure 3D) using decreasing concentration of gammaglobin peptides and recombinant fragments.

Figures 4A-4B present the results of monoclonal antibody characterization by FACS analysis. Each monoclonal antibody was used to detect mammaglobin expression in MDA-MB-415 cells. Samples were fixed in 2% formaldehyde and permeabilized with 0.5% saponin. MCF-7 cells do not express mammaglobin and were 5 used as a negative control.

Figure 5 presents Western blot detection of mammaglobin by each monoclonal antibody. SDS-PAGE was performed on media in which MDA-MB-415 cells were grown, MDA-MB-415 cell lysate and bacterially expressed recombinant mammaglobin, as indicated. Mammaglobin expression was detected with the indicated 10 antibody.

Figure 6 is a table showing mammaglobin expression in breast tissue, but not in other tissues tested. Mammaglobin expression in various tissues was evaluated by immunohistochemistry analysis using a combination of 29C11 and 31A5 rabbit monoclonal antibodies.

Figures 7A-7C are graphs illustrating the results of sandwich assays performed using the indicated rabbit monoclonal antibodies to detect mammaglobin in lysates and supernatants of MB415 cells.

Figure 8 is a graph showing the standard curve for a sandwich assay using the polyclonal anti-967 serum in combination with the monoclonal antibody 2D3 20 biotinylated.

Figure 9 is a table showing the results of sandwich assays using the representative indicated antibodies to detect mammaglobin in patients with and without breast cancer.

Figure 10 presents the human mammaglobin amino acid sequence (SEQ ID 25 NO: 27), with underlined and bold peptide regions (SEQ ID Nos: 29-36) used for epitope mapping studies.

Figures 11A and 11B are graphs illustrating the recognition of CD4 T cell lines for mammaglobin and various portions thereof, as indicated. Figure 11A shows T cell proliferation of three different CD4 T cell lines in response to various proteins and

peptides. Figure 11B shows interferon- γ production by the same cell lines in response to the same proteins and peptides.

Figure 12 presents the human mammaglobin amino acid sequence (SEQ ID NO: 27), along with peptide regions (SEQ ID Nos: 37-45) used for CD4 $^{+}$ and T cell epitope 5 mapping studies.

Figures 13A-13C are graphs illustrating the recognition of JurkatA2Kb cells pulsed with mgb-1 by CTL from HLA A2 transgenic mice immunized with mgb 1. CTL from three different mice were tested at different effector:target ratios, as indicated. Each 10 figure shows the percent specific lysis of cells that are (solid circles) and are not (open circles) pulsed with mgb-1.

Figures 14A-14C are graphs illustrating the recognition of JurkatA2Kb cells pulsed with mgb-1 (triangles) or expressing full length mammaglobin (mammaglobin) by CTL from HLA A2 transgenic mice immunized with mgb 1. CTL from three different mice were tested at different effector:target ratios, as indicated. In each figure, the percent 15 specific lysis of cells that do not express mgb-1 or mammaglobin is represented by circles.

Figure 15 is a histogram showing the tissue distribution for mammaglobin. Copies of mammaglobin per ng β -actin are shown for a variety of normal and tumor tissues, as indicated.

Figure 16 is a graph showing the number of copies of mammaglobin 20 message in the breast cancer cell line MB415 as a function of the amount of cells.

Figure 17 is a histogram showing the detection of mammaglobin in epithelial cells isolated, using the Dynal isolation method, from the peripheral blood of patients with metastatic breast cancer compared to similar isolates from normal blood samples. Copies of mammaglobin per ng β -actin are shown for thirty three metastatic and 25 11 normal samples, as indicated.

Figure 18 is a table showing the results of experiments in which T cell proliferation was measured. The T cells of indicated cell lines were primed with a priming peptide as indicated, then incubated with one of the following: 1A-7A (a pool of seven peptides); 3A (peptide 3A); 5A (peptide 5A); 7A (peptide 7A); mgb B 5A (a peptide

corresponding to 5A, but from the same region of the mammaglobin B sequence); Hmamm10; Hmamm1; Hmamm6.

Figure 19 shows three bar graphs in which cell lines 13, 14 and 27 were stimulated with one of the following: medium, recombinant mammoglobin, native 5 mammoglobin, deglycosylated native mammoglobin, MB415 lysate, and SKOV3 lysate. SI=Stimulation Index.

Figure 20 is a schematic diagram of recombinant full-length mammoglobin.

Figure 21 shows the results of SDS-PAGE analysis of recombinant full-length mammoglobin expression, stained with Coomassie blue.

10 DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention is generally directed to compositions and methods for the therapy, diagnosis and monitoring of cancer, such as breast cancer. The compositions described herein may include mammoglobin polynucleotides, polypeptides, epitopes or antibodies that specifically recognize such epitopes. The present 15 invention is based, in part, on the discovery of certain specific epitopes of human mammoglobin, and antibodies that bind such epitopes. The invention is further based, in part, on the discovery of antibodies that bind mammoglobin in a glycosylation-sensitive manner. Other methods described herein employ techniques for detecting mammoglobin nucleic acid in patient blood, or fractions thereof. These discoveries, within the context of 20 the present invention, permit the generation of antibodies suited for diagnostic purposes, improved therapies for breast cancer, as well as diagnostic methods that can be based on the detection of mammoglobin RNA in blood permits sensitive diagnosis of breast cancer.

MAMMAGLOBIN POLYNUCLEOTIDES

25 The diagnostic methods provided herein generally employ mammoglobin polynucleotides (e.g., oligonucleotides) as probes or primers to detect the level of mammoglobin nucleic acid in a sample obtained from a patient. A mammoglobin oligonucleotide may encode a portion of a mammoglobin protein (e.g., at least 15, 30 or 45

consecutive nucleotides). Oligonucleotides complementary to any such sequences are also encompassed by the present invention. Polynucleotides may be single-stranded (coding or antisense) or double-stranded, and may be DNA (genomic, cDNA or synthetic) or RNA molecules. RNA molecules include HnRNA molecules, which contain introns and 5 correspond to a DNA molecule in a one-to-one manner, and mRNA molecules, which do not contain introns. Additional coding or non-coding sequences may, but need not, be present within a polynucleotide of the present invention, and a polynucleotide may, but need not, be linked to other molecules and/or support materials.

Polynucleotides may comprise a native sequence (i.e., a portion of 10 endogenous gammaglobin) or may comprise a variant of such a sequence. Polynucleotide variants may contain one or more substitutions, additions, deletions and/or insertions such that the ability of the polynucleotide to hybridize to a gammaglobin polynucleotide under assay conditions is not substantially diminished. Preferably, such polynucleotide variants are capable of hybridizing under moderately stringent conditions to a naturally occurring 15 DNA sequence encoding a native gammaglobin (or a complementary sequence). Suitable moderately stringent conditions include prewashing in a solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridizing at 50°C-65°C, 5 X SSC, overnight; followed by washing twice at 65°C for 20 minutes with each of 2X, 0.5X and 0.2X SSC containing 0.1% SDS.

20 Polynucleotides may be prepared using any of a variety of techniques. For example, polynucleotides may be amplified from cDNA prepared from cells expressing gammaglobin, such as breast tumor cells. Such polynucleotides may be amplified via polymerase chain reaction (PCR). For this approach, sequence-specific primers may be designed based on known gammaglobin sequences, and may be purchased or synthesized.

25 A portion of a coding sequence or a complementary sequence may be designed as a probe or primer to detect gene expression. Probes may be labeled by a variety of reporter groups, such as radionuclides and enzymes, and are preferably at least 10 nucleotides in length, more preferably at least 20 nucleotides in length and still more

preferably at least 30 nucleotides in length. Primers are preferably 22-30 nucleotides in length.

Any polynucleotide may be further modified to increase stability in vivo. Possible modifications include, but are not limited to, the addition of flanking sequences at 5 the 5' and/or 3' ends; the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages in the backbone; and/or the inclusion of nontraditional bases such as inosine, queosine and wybutoxine, as well as acetyl- methyl-, thio- and other modified forms of adenine, cytidine, guanine, thymine and uridine.

Nucleotide sequences as described herein may be joined to a variety of other 10 nucleotide sequences using established recombinant DNA techniques. For example, a polynucleotide may be cloned into any of a variety of cloning vectors, including plasmids, phagemids, lambda phage derivatives and cosmids. Vectors of particular interest include expression vectors, replication vectors, probe generation vectors and sequencing vectors. In general, a vector will contain an origin of replication functional in at least one organism, 15 convenient restriction endonuclease sites and one or more selectable markers. Other elements will depend upon the desired use, and will be apparent to those of ordinary skill in the art.

MAMMAGLOBIN EPITOPES AND POLYPEPTIDES

20 Within the context of the present invention, polypeptides comprise at least one mammaglobin epitope, or a variant thereof. An "epitope" is a portion of mammaglobin to which one or more antibodies within an anti-mammaglobin antiserum specifically binds, or with which one or more mammaglobin-specific T cells specifically reacts, as described herein. An epitope may, but need not, be specifically bound by an antibody in a 25 glycosylation-sensitive manner (i.e., the antibody may bind to a glycosylated epitope, to a deglycosylated epitope or to both). Polypeptides comprising a mammaglobin epitope generally comprise at least 7 consecutive amino acid residues of human mammaglobin, and preferably 9-30 consecutive amino acid residues of human mammaglobin. It should be noted that the size of an epitope may vary depending on whether the epitope is recognized

by CD4⁺ T cells, CD8⁺ T cells or antibodies. In general, however, a 9-amino acid sequence is sufficient for TCL recognition. Polypeptides as described herein may be of any length. Additional sequences derived from the native protein and/or heterologous sequences may be present, and such sequences may (but need not) possess further 5 immunogenic or antigenic properties.

Certain preferred epitopes comprise one of the following sequences, or a portion thereof that comprises at least 7, preferably at least 9 and more preferably at least 15 consecutive amino acid residues of such a sequence:

IDEK~~ECFLN~~QTD~~ETL~~SNVE (Pro2; SEQ ID NO: 1);
10 TTNA~~I~~DELKECFLNQ (Pro2-3; SEQ ID NO: 2);
SQH~~CY~~AGSGCP~~LLEN~~VI~~SKTI~~ (Pro5; SEQ ID NO: 3);
EYKELLQEFIDD~~NATTNAID~~ (peptide 5A; SEQ ID NO: 4) or
KLLMVLMLA (mgb 1; SEQ ID NO: 5).

Other preferred epitopes comprise a glycosylation site of gammaglobin. Such epitopes are 15 particularly useful for the generation of antibodies that specifically bind to glycosylated gammaglobin. Two such sites are the N-linked glycosylation sites asparagine (Asp)-53 (QEFIDDNATTNAI) (SEQ ID NO: 6) and Asp-68 (LKECFLNQTDETL) (SEQ ID NO: 7). Other such sites may be readily identified using, for example, an antibody library comprising antibodies to different glycosylation combinations. The binding of such 20 antibodies to native gammaglobin from breast carcinoma cell lines may be assayed using conventional ELISA and blotting techniques. Established biochemical techniques may also be used to identify other gammaglobin glycosylation sites.

As noted above, a polypeptide may comprise a variant of a native gammaglobin epitope. A "variant," as used herein, differs from a native epitope in one or 25 more substitutions, deletions, additions and/or insertions, such that the ability of the variant to be bound by an antibody specific for the epitope is not substantially diminished. In other words, the ability of a variant to react with epitope-specific antisera or isolated antibodies may be enhanced or unchanged, relative to the native protein, or may be diminished by less than 50%, and preferably less than 20%, relative to the native protein. Such variants may

generally be identified by modifying an epitope as provided herein and evaluating the reactivity of the modified epitope with epitope-specific antibodies or antisera as described herein. Preferred variants include those in which substitutions are made at no more than 20% of the residues in the epitope.

5 Preferably, a variant contains conservative substitutions. A "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydrophobic nature of the polypeptide to be substantially unchanged. Amino acid substitutions may generally be made on the basis of similarity in
10 polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine and valine; glycine and alanine; asparagine and glutamine; and serine, threonine,
15 phenylalanine and tyrosine. Other groups of amino acids that may represent conservative changes include: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. A variant may also, or alternatively, contain nonconservative changes. Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal
20 influence on the immunogenicity, secondary structure and hydrophobic nature of the polypeptide.

As noted above, polypeptides may comprise a signal (or leader) sequence at the N-terminal end of the protein, which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification or identification of the polypeptide (e.g., poly-His), or to enhance binding of the polypeptide to a solid support. For example, a polypeptide may be conjugated to an immunoglobulin Fc region.

Polypeptides may be prepared using any of a variety of well known techniques. Recombinant polypeptides may be readily prepared from mammaglobin DNA

sequences using any of a variety of expression vectors known to those of ordinary skill in the art. Expression may be achieved in any appropriate host cell that has been transformed or transfected with an expression vector containing a DNA molecule that encodes a recombinant polypeptide. Suitable host cells include prokaryotes, yeast and higher 5 eukaryotic cells. Preferably, the host cells employed are *E. coli*, yeast or a mammalian cell line such as COS or CHO. Supernatants from suitable host/vector systems that secrete recombinant protein or polypeptide into culture media may be first concentrated using a commercially available filter. Following concentration, the concentrate may be applied to a suitable purification matrix such as an affinity matrix or an ion exchange resin. Finally, 10 one or more reverse phase HPLC steps can be employed to further purify a recombinant polypeptide.

Polypeptides having fewer than about 100 amino acids, and generally fewer than about 50 amino acids, may also be generated by synthetic means, using techniques well known to those of ordinary skill in the art. For example, such polypeptides may be 15 synthesized using any of the commercially available solid-phase techniques, such as the Merrifield solid-phase synthesis method, where amino acids are sequentially added to a growing amino acid chain. See Merrifield, *J. Am. Chem. Soc.* 85:2149-2146, 1963. Equipment for automated synthesis of polypeptides is commercially available from suppliers such as Applied BioSystems, Inc. (Foster City, CA), and may be operated 20 according to the manufacturer's instructions.

Within related aspects, polynucleotides that encode a polypeptide as provided herein are provided. In general, polypeptides and polynucleotides as described herein are isolated. An "isolated" polypeptide or polynucleotide is one that is removed from its original environment. For example, a naturally-occurring protein is isolated if it is 25 separated from some or all of the coexisting materials in the natural system. Preferably, such polypeptides are at least about 90% pure, more preferably at least about 95% pure and most preferably at least about 99% pure. A polynucleotide is considered to be isolated if, for example, it is cloned into a vector that is not a part of the natural environment.

ANTIBODIES AND FRAGMENTS THEREOF

The present invention further provides agents, such as antibodies and antigen-binding fragments thereof, that specifically bind to a mammaglobin epitope. As used herein, an antibody, or antigen-binding fragment thereof, is said to "specifically bind" 5 to a mammaglobin epitope if it reacts at a detectable level (within, for example, an ELISA) with the epitope, and does not react detectably with unrelated proteins under similar conditions. Preferred antibodies bind detectably to an epitope of mammaglobin, but do not bind detectably to other portions of mammaglobin that do not overlap with the epitope (or that overlap by less than five amino acid residues). As used herein, "binding" refers to a 10 noncovalent association between two separate molecules such that a complex is formed. The ability to bind may be evaluated by, for example, determining a binding constant for the formation of the complex. The binding constant is the value obtained when the concentration of the complex is divided by the product of the component concentrations. In general, two compounds are said to "bind," in the context of the present invention, when 15 the binding constant for complex formation exceeds about 10^3 L/mol. The binding constant maybe determined using methods well known in the art.

Binding agents may be further capable of differentiating between patients with and without a cancer, such as breast cancer, using the representative assays provided herein. In other words, antibodies or other binding agents that bind to a mammaglobin 20 epitope will generate a signal indicating the presence of a cancer in at least about 20% of patients with the disease, and will generate a negative signal indicating the absence of the disease in at least about 90% of individuals without the cancer. To determine whether a binding agent satisfies this requirement, biological samples (e.g., blood, sera, urine and/or tumor biopsies) from patients with and without a cancer (as determined using standard 25 clinical tests) may be assayed as described herein for the presence of polypeptides that bind to the binding agent. It will be apparent that a statistically significant number of samples with and without the disease should be assayed. Each binding agent should satisfy the above criteria; however, those of ordinary skill in the art will recognize that binding agents may be used in combination to improve sensitivity.

Any agent that satisfies the above requirements may be a binding agent. For example, a binding agent may be a ribosome, with or without a peptide component, and RNA molecule or a polypeptide. In a preferred embodiment, a binding agent is an antibody or an antigen-binding fragment thereof. Antibodies may be prepared by any of a variety of 5 techniques known to those of ordinary skill in the art. *See, e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. In general, antibodies can be produced by cell culture techniques, including the generation of monoclonal antibodies as described herein, or via transfection of antibody genes into suitable bacterial or mammalian cell hosts, in order to allow for the production of 10 recombinant antibodies. In one technique, an immunogen comprising the polypeptide is initially injected into any of a wide variety of mammals (*e.g.*, mice, rats, rabbits, sheep or goats). In this step, the polypeptides of this invention may serve as the immunogen without modification. Alternatively, particularly for relatively short polypeptides, a superior 15 immune response may be elicited if the polypeptide is joined to a carrier protein, such as bovine serum albumin or keyhole limpet hemocyanin. The immunogen is injected into the animal host, preferably according to a predetermined schedule incorporating one or more booster immunizations, and the animals are bled periodically. Polyclonal antibodies specific for the polypeptide may then be purified from such antisera by, for example, affinity chromatography using the polypeptide coupled to a suitable solid support.

20 Monoclonal antibodies specific for an antigenic polypeptide of interest may be prepared, for example, using the technique of Kohler and Milstein, *Eur. J. Immunol.* 6:511-519, 1976, and improvements thereto. Briefly, these methods involve the preparation of immortal cell lines capable of producing antibodies having the desired specificity (*i.e.*, reactivity with the polypeptide of interest). Such cell lines may be 25 produced, for example, from spleen cells obtained from an animal immunized as described above. The spleen cells are then immortalized by, for example, fusion with a myeloma cell fusion partner, preferably one that is syngeneic with the immunized animal. A variety of fusion techniques may be employed. For example, the spleen cells and myeloma cells may be combined with a nonionic detergent for a few minutes and then plated at low density on

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a selective medium that supports the growth of hybrid cells, but not myeloma cells. A preferred selection technique uses HAT (hypoxanthine, aminopterin, thymidine) selection. After a sufficient time, usually about 1 to 2 weeks, colonies of hybrids are observed. Single colonies are selected and their culture supernatants tested for binding activity against 5 the polypeptide. Hybridomas having high reactivity and specificity are preferred.

Monoclonal antibodies may be isolated from the supernatants of growing hybridoma colonies. In addition, various techniques may be employed to enhance the yield, such as injection of the hybridoma cell line into the peritoneal cavity of a suitable vertebrate host, such as a mouse. Monoclonal antibodies may then be harvested from the 10 ascites fluid or the blood. Contaminants may be removed from the antibodies by conventional techniques, such as chromatography, gel filtration, precipitation, and extraction. The polypeptides of this invention may be used in the purification process in, for example, an affinity chromatography step.

Certain preferred monoclonal antibodies specifically bind to an epitope 15 sequence recited above (Pro2, Pro2-3, Pro5, peptide 5A or mgb 1). Such antibodies include the rabbit antibodies designated 29C11, 6A1, 2D3 and 16D8 and the mouse antibody designated 197-1H11 herein. Other preferred antibodies bind to other sequences, such as conformationally dependent sequences. Such antibodies include those designated 31-1H7, 32-1G11, 304-1A5 and 98-1F4 herein. Other preferred antibodies bind to a glycosylation 20 site of gammaglobulin with an affinity that is dependent on glycosylation. For example, certain antibodies specifically bind to glycosylated gammaglobulin (*i.e.*, require glycosylation of a particular glycosylation site for optimal binding). As used herein, an antibody, or antigen binding fragment thereof, specifically binds to glycosylated gammaglobulin if it binds to a glycosylated gammaglobulin with an affinity that is at least 25 two-fold, preferably at least five-fold, greater than the affinity with which it binds deglycosylated gammaglobulin (gammaglobulin that is enzymatically deglycosylated, using well known techniques, so as to remove substantially all glycosylation). Glycosylation results when oligosaccharide units are attached to the protein via asparagine (N-linked) or serine and threonine residues (O-linked). Compared to normal cells, protein glycosylation

is often altered in tumor cells. This difference in protein glycosylation can be exploited to provide a tumor-specific antibody for diagnostic purposes (e.g., for the diagnosis of breast cancer). This is particularly true for heavily glycosylated proteins, such as mammaglobin. Although the predicted molecular weight of mammaglobin is 9.2 kDa, the mature form of 5 this protein expressed in breast carcinoma cells runs at a molecular weight of approximately 18-25kDa. It has been found, within the context of the present invention, that the additional molecular weight of mammaglobin is due to the attachment of oligosaccharides. Thus, roughly one half or more of the molecular weight of mammaglobin is due to glycosylation.

10 Within certain embodiments, the use of antigen-binding fragments of antibodies may be preferred. Such fragments include Fab fragments, which may be prepared using standard techniques. Briefly, immunoglobulins may be purified from rabbit serum by affinity chromatography on Protein A bead columns (Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988) and digested by 15 papain to yield Fab and Fc fragments. The Fab and Fc fragments may be separated by affinity chromatography on protein A bead columns.

Monoclonal antibodies of the present invention may be coupled to one or more therapeutic agents. Suitable agents in this regard include radionuclides, differentiation inducers, drugs, toxins, and derivatives thereof. Preferred radionuclides 20 include ⁹⁰Y, ¹²³I, ¹²⁵I, ¹³¹I, ¹⁸⁶Re, ¹⁸⁸Re, ²¹¹At, and ²¹²Bi. Preferred drugs include methotrexate, and pyrimidine and purine analogs. Preferred differentiation inducers include phorbol esters and butyric acid. Preferred toxins include ricin, abrin, diphteria toxin, cholera toxin, gelonin, *Pseudomonas* exotoxin, *Shigella* toxin, and pokeweed antiviral protein.

25 A therapeutic agent may be coupled (e.g., covalently bonded) to a suitable monoclonal antibody either directly or indirectly (e.g., via a linker group). A direct reaction between an agent and an antibody is possible when each possesses a substituent capable of reacting with the other. For example, a nucleophilic group, such as an amino or sulphhydryl group, on one may be capable of reacting with a carbonyl-containing group,

such as an anhydride or an acid halide, or with an alkyl group containing a good leaving group (*e.g.*, a halide) on the other.

Alternatively, it may be desirable to couple a therapeutic agent and an antibody via a linker group. A linker group can function as a spacer to distance an antibody 5 from an agent in order to avoid interference with binding capabilities. A linker group can also serve to increase the chemical reactivity of a substituent on an agent or an antibody, and thus increase the coupling efficiency. An increase in chemical reactivity may also facilitate the use of agents, or functional groups on agents, which otherwise would not be possible.

10 It will be evident to those skilled in the art that a variety of bifunctional or polyfunctional reagents, both homo- and hetero-functional (such as those described in the catalog of the Pierce Chemical Co., Rockford, IL), may be employed as the linker group. Coupling may be effected, for example, through amino groups, carboxyl groups, sulphhydryl groups or oxidized carbohydrate residues. There are numerous references describing such 15 methodology, *e.g.*, U.S. Patent No. 4,671,958, to Rodwell et al.

Where a therapeutic agent is more potent when free from the antibody portion of the immunoconjugates of the present invention, it may be desirable to use a linker group which is cleavable during or upon internalization into a cell. A number of different cleavable linker groups have been described. The mechanisms for the 20 intracellular release of an agent from these linker groups include cleavage by reduction of a disulfide bond (*e.g.*, U.S. Patent No. 4,489,710, to Spitzer), by irradiation of a photolabile bond (*e.g.*, U.S. Patent No. 4,625,014, to Senter et al.), by hydrolysis of derivatized amino acid side chains (*e.g.*, U.S. Patent No. 4,638,045, to Kohn et al.), by serum complement-mediated hydrolysis (*e.g.*, U.S. Patent No. 4,671,958, to Rodwell et al.), and acid-catalyzed 25 hydrolysis (*e.g.*, U.S. Patent No. 4,569,789, to Blattler et al.).

It may be desirable to couple more than one agent to an antibody. In one embodiment, multiple molecules of an agent are coupled to one antibody molecule. In another embodiment, more than one type of agent may be coupled to one antibody. Regardless of the particular embodiment, immunoconjugates with more than one agent

may be prepared in a variety of ways. For example, more than one agent may be coupled directly to an antibody molecule, or linkers that provide multiple sites for attachment can be used. Alternatively, a carrier can be used.

A carrier may bear the agents in a variety of ways, including covalent
5 bonding either directly or via a linker group. Suitable carriers include proteins such as albumins (e.g., U.S. Patent No. 4,507,234, to Kato et al.), peptides and polysaccharides such as aminodextran (e.g., U.S. Patent No. 4,699,784, to Shih et al.). A carrier may also bear an agent by noncovalent bonding or by encapsulation, such as within a liposome vesicle (e.g., U.S. Patent Nos. 4,429,008 and 4,873,088). Carriers specific for radionuclide
10 agents include radiohalogenated small molecules and chelating compounds. For example, U.S. Patent No. 4,735,792 discloses representative radiohalogenated small molecules and their synthesis. A radionuclide chelate may be formed from chelating compounds that include those containing nitrogen and sulfur atoms as the donor atoms for binding the metal, or metal oxide, radionuclide. For example, U.S. Patent No. 4,673,562, to Davison
15 et al. discloses representative chelating compounds and their synthesis.

A variety of routes of administration for the antibodies and immunoconjugates may be used. Typically, administration will be intravenous, intramuscular, subcutaneous or in the bed of a resected tumor. It will be evident that the precise dose of the antibody/immunoconjugate will vary depending upon the antibody
20 used, the antigen density on the tumor, and the rate of clearance of the antibody.

T CELLS

Immunotherapeutic compositions may also, or alternatively, comprise T cells that recognize mammaglobin. Such cells may generally be prepared *in vitro* or *ex*
25 *vivo*, using standard procedures. For example, T cells may be isolated from bone marrow, peripheral blood, or a fraction of bone marrow or peripheral blood of a patient, using a commercially available cell separation system, such as the Isolex™ System, available from
Nexell Therapeutics, Inc. (Irvine, CA; see also U.S. Patent No. 5,240,856; U.S. Patent No.
5,215,926; WO 89/06280; WO 91/16116 and WO 92/07243). Alternatively, T cells may

be derived from related or unrelated humans, non-human mammals, cell lines or cultures. Briefly, T cells, which may be isolated from a patient or a related or unrelated donor by routine techniques (such as by Ficoll/Hypaque density gradient centrifugation of peripheral blood lymphocytes), are incubated with a mammaglobin polypeptide. For example, T cells 5 may be incubated *in vitro* for 2-9 days (typically 4 days) at 37°C with a mammaglobin polypeptide (e.g., 5 to 25 µg/ml) or cells synthesizing a comparable amount of mammaglobin polypeptide. It may be desirable to incubate a separate aliquot of a T cell sample in the absence of mammaglobin polypeptide to serve as a control.

T cells may be stimulated with a mammaglobin polypeptide, polynucleotide 10 encoding a mammaglobin polypeptide and/or an antigen presenting cell (APC) that expresses such a polypeptide. Such stimulation is performed under conditions and for a time sufficient to permit the generation of T cells that are specific for the polypeptide. Preferably, a mammaglobin polypeptide or polynucleotide is present within a delivery vehicle, such as a microsphere, to facilitate the generation of specific T cells.

15 T cells are considered to be specific for a mammaglobin polypeptide if the T cells specifically proliferate, secrete cytokines or kill target cells coated with the polypeptide or expressing a gene encoding the polypeptide. T cell specificity may be evaluated using any of a variety of standard techniques. For example, within a chromium release assay or proliferation assay, a stimulation index of more than two fold increase in 20 lysis and/or proliferation, compared to negative controls, indicates T cell specificity. Such assays may be performed, for example, as described in Chen et al., *Cancer Res.* 54:1065-1070, 1994. Alternatively, detection of the proliferation of T cells may be accomplished by a variety of known techniques. For example, T cell proliferation can be detected by measuring an increased rate of DNA synthesis (e.g., by pulse-labeling cultures of T cells 25 with tritiated thymidine and measuring the amount of tritiated thymidine incorporated into DNA). Other ways to detect T cell proliferation include measuring increases in interleukin-2 (IL-2) production, Ca²⁺ flux, or dye uptake, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium. Alternatively, synthesis of lymphokines (such as interferon-gamma) can be measured or the relative number of T cells that can respond to a mammaglobin

polypeptide may be quantified. Contact with a mammaglobin polypeptide (100 ng/ml - 100 μ g/ml, preferably 200 ng/ml - 25 μ g/ml) for 3 - 7 days should result in at least a two fold increase in proliferation of the T cells. Contact as described above for 2-3 hours should result in activation of the T cells, as measured using standard cytokine assays in 5 which a two fold increase in the level of cytokine release (*e.g.*, TNF or IFN- γ) is indicative of T cell activation (*see* Coligan et al., Current Protocols in Immunology, vol. 1, Wiley Interscience (Greene 1998)). Mammaglobin-specific T cells may be expanded using standard techniques. Within preferred embodiments, the T cells are derived from a patient, a related donor or an unrelated donor, and are administered to the patient following 10 stimulation and expansion.

T cells that have been activated in response to a mammaglobin polypeptide, polynucleotide or polypeptide-expressing APC may be CD4 $^{+}$ and/or CD8 $^{+}$. Specific activation of CD4 $^{+}$ or CD8 $^{+}$ T cells may be detected in a variety of ways. Methods for detecting specific T cell activation include detecting the proliferation of T cells, the 15 production of cytokines (*e.g.*, lymphokines), or the generation of cytolytic activity (*i.e.*, generation of cytotoxic T cells specific for mammaglobin). For CD4 $^{+}$ T cells, a preferred method for detecting specific T cell activation is the detection of the proliferation of T cells. For CD8 $^{+}$ T cells, a preferred method for detecting specific T cell activation is the detection of the generation of cytolytic activity.

20 For therapeutic purposes, CD4 $^{+}$ or CD8 $^{+}$ T cells that proliferate in response to a mammaglobin polypeptide, polynucleotide or APC can be expanded in number either *in vitro* or *in vivo*. Proliferation of such T cells *in vitro* may be accomplished in a variety of ways. For example, the T cells can be re-exposed to a mammaglobin polypeptide (*e.g.*, a short peptide corresponding to an immunogenic portion of such a polypeptide) with or 25 without the addition of T cell growth factors, such as interleukin-2, and/or stimulator cells that synthesize a mammaglobin polypeptide. The addition of stimulator cells is preferred where generating CD8 $^{+}$ T cell responses. T cells can be grown to large numbers *in vitro* with retention of specificity in response to intermittent restimulation with mammaglobin polypeptide. Briefly, for *in vitro* stimulation, lymphocytes may be placed in a vessel with

media containing human serum, gammaglobulin protein or peptide and cytokines such as IL-2, IL-10 and IL-7. Cells may be incubated for seven to fourteen days and then restimulated in a similar manner using autologous antigen presenting cells, gammaglobulin protein or peptide and cytokines. Antigen specific T cells may also be expanded *in vitro* using either 5 antigen or a mitogen or non-specific stimulator such as α -CD3 or PHA.

Alternatively, one or more T cells that proliferate in the presence of gammaglobulin polypeptide can be expanded in number by cloning. Methods for cloning cells are well known in the art, and include limiting dilution. Responder T cells may be purified from the peripheral blood of sensitized patients by density gradient centrifugation 10 and sheep red cell rosetting and established in culture by stimulating with the nominal antigen in the presence of irradiated autologous filler cells. In order to generate CD4 $^{+}$ T cell lines, gammaglobulin polypeptide is used as the antigenic stimulus and APC derived from autologous peripheral blood lymphocytes (PBL) or lymphoblastoid cell lines (LCL) immortalized by infection with Epstein Barr virus are used as antigen presenting cells. In 15 order to generate CD8 $^{+}$ T cell lines, autologous antigen-presenting cells transfected with an expression vector that produces gammaglobulin polypeptide may be used as stimulator cells. Established T cell lines may be cloned following antigen stimulation by plating stimulated T cells at a frequency of 0.5 cells per well in 96-well flat-bottom plates with 1 x 10⁶ irradiated PBL or LCL cells and recombinant interleukin-2 (rIL2) (50 U/ml). Wells with 20 established clonal growth may be identified at approximately 2-3 weeks after initial plating and restimulated with appropriate antigen in the presence of autologous antigen-presenting cells, then subsequently expanded by the addition of low doses of rIL2 (10 U/ml) 2-3 days following antigen stimulation. T cell clones may be maintained in 24-well plates by periodic restimulation with antigen and rIL2 approximately every two weeks. Cloned 25 and/or expanded cells may be administered back to the patient as described, for example, by Chang et al., *Crit. Rev. Oncol. Hematol.* 22:213, 1996.

Within certain embodiments, allogeneic T-cells may be primed (*i.e.*, sensitized to gammaglobulin) *in vivo* and/or *in vitro*. Such priming may be achieved by contacting T cells with a gammaglobulin polypeptide, a polynucleotide encoding such a

polypeptide or a cell producing such a polypeptide under conditions and for a time sufficient to permit the priming of T cells. In general, T cells are considered to be primed if, for example, contact with a gammaglobulin polypeptide results in proliferation and/or activation of the T cells, as measured by standard proliferation, chromium release and/or 5 cytokine release assays as described herein. A stimulation index of more than two fold increase in proliferation or lysis, and more than three fold increase in the level of cytokine, compared to negative controls, indicates T-cell specificity. Cells primed *in vitro* may be employed, for example, within a bone marrow transplantation or as donor lymphocyte infusion.

10

PHARMACEUTICAL COMPOSITIONS AND VACCINES

Within certain aspects, polypeptides, polynucleotides, T cells and/or binding agents described herein may be incorporated into pharmaceutical compositions or immunogenic compositions (*i.e.*, vaccines). Alternatively, a pharmaceutical composition 15 may comprise an antigen-presenting cell (*e.g.*, a dendritic cell) transfected with a gammaglobulin polynucleotide such that the antigen presenting cell expresses a gammaglobulin polypeptide. Pharmaceutical compositions comprise one or more such compounds and a physiologically acceptable carrier. Vaccines may comprise one or more such compounds and an immunostimulant. An immunostimulant may be any substance 20 that enhances or potentiates an immune response (antibody- and/or cell-mediated) to an exogenous antigen. Examples of immunostimulants include adjuvants, biodegradable microspheres (*e.g.*, polylactic galactide) and liposomes (into which the compound is incorporated; *see e.g.*, Fullerton, U.S. Patent No. 4,235,877). Vaccine preparation is generally described in, for example, M.F. Powell and M.J. Newman, eds., "Vaccine Design 25 (the subunit and adjuvant approach)," Plenum Press (NY, 1995). Pharmaceutical compositions and vaccines within the scope of the present invention may also contain other compounds, which may be biologically active or inactive. For example, one or more immunogenic portions of other tumor antigens may be present, either incorporated into a fusion polypeptide or as a separate compound, within the composition or vaccine.

A pharmaceutical composition or vaccine may contain DNA encoding one or more of the polypeptides as described above, such that the polypeptide is generated *in situ*. As noted above, the DNA may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid expression systems, 5 bacteria and viral expression systems and mammalian expression systems. Numerous gene delivery techniques are well known in the art, such as those described by Rolland, *Crit. Rev. Therap. Drug Carrier Systems* 15:143-198, 1998, and references cited therein. Appropriate nucleic acid expression systems contain the necessary DNA sequences for expression in the patient (such as a suitable promoter and terminating signal). Bacterial 10 delivery systems involve the administration of a bacterium (such as *Bacillus-Calmette-Guerrin*) that expresses an immunogenic portion of the polypeptide on its cell surface or secretes such an epitope. In a preferred embodiment, the DNA may be introduced using a viral expression system (e.g., vaccinia or other pox virus, retrovirus, or adenovirus), which may involve the use of a non-pathogenic (defective), replication competent virus. Suitable 15 systems are disclosed, for example, in Fisher-Hoch et al., *Proc. Natl. Acad. Sci. USA* 86:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci.* 569:86-103, 1989; Flexner et al., *Vaccine* 8:17-21, 1990; U.S. Patent Nos. 4,603,112, 4,769,330, and 5,017,487; WO 89/01973; U.S. Patent No. 4,777,127; GB 2,200,651; EP 0,345,242; WO 91/02805; Berkner, *Biotechniques* 6:616-627, 1988; Rosenfeld et al., *Science* 252:431-434, 1991; 20 Kolls et al., *Proc. Natl. Acad. Sci. USA* 91:215-219, 1994; Kass-Eisler et al., *Proc. Natl. Acad. Sci. USA* 90:11498-11502, 1993; Guzman et al., *Circulation* 88:2838-2848, 1993; and Guzman et al., *Cir. Res.* 73:1202-1207, 1993. Techniques for incorporating DNA into such expression systems are well known to those of ordinary skill in the art. The DNA may also be "naked," as described, for example, in Ulmer et al., *Science* 259:1745-1749, 25 1993 and reviewed by Cohen, *Science* 259:1691-1692, 1993. The uptake of naked DNA may be increased by coating the DNA onto biodegradable beads, which are efficiently transported into the cells. It will be apparent that a vaccine may comprise both a polynucleotide and a polypeptide component. Such vaccines may provide for an enhanced immune response.

It will be apparent that a vaccine may contain pharmaceutically acceptable salts of the polynucleotides and polypeptides provided herein. Such salts may be prepared from pharmaceutically acceptable non-toxic bases, including organic bases (e.g., salts of primary, secondary and tertiary amines and basic amino acids) and inorganic bases (e.g., 5 sodium, potassium, lithium, ammonium, calcium and magnesium salts).

While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will vary depending on the mode of administration. Compositions of the present invention may be formulated for any appropriate manner of administration, including for example, topical, 10 oral, nasal, intravenous, intracranial, intraperitoneal, subcutaneous or intramuscular administration. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and 15 magnesium carbonate, may be employed. Biodegradable microspheres (e.g., polylactate polyglycolate) may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Patent Nos. 4,897,268; 5,075,109; 5,928,647; 5,811,128; 5,820,883; 5,853,763; 5,814,344 and 5,942,252.

20 Such compositions may also comprise buffers (e.g., neutral buffered saline or phosphate buffered saline), carbohydrates (e.g., glucose, mannose, sucrose or dextrans), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, bacteriostats, chelating agents such as EDTA or glutathione, adjuvants (e.g., aluminum hydroxide), solutes that render the formulation isotonic, hypotonic or weakly hypertonic with the blood 25 of a recipient, suspending agents, thickening agents and/or preservatives. Alternatively, compositions of the present invention may be formulated as a lyophilizate. Compounds may also be encapsulated within liposomes using well known technology.

Any of a variety of immunostimulants may be employed in the vaccines of this invention. For example, an adjuvant may be included. Most adjuvants contain a

substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bordetella pertussis* or *Mycobacterium tuberculosis* derived proteins. Suitable adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete 5 Adjuvant (Difco Laboratories, Detroit, MI); Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ); AS-2 (SmithKline Beecham, Philadelphia, PA); aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; 10 monophosphoryl lipid A and quill A. Cytokines, such as GM-CSF or interleukin-2, -7, or -12, may also be used as adjuvants.

Within the vaccines provided herein, the adjuvant composition is preferably designed to induce an immune response predominantly of the Th1 type. High levels of Th1-type cytokines (e.g., IFN- γ , TNF α , IL-2 and IL-12) tend to favor the induction of cell 15 mediated immune responses to an administered antigen. In contrast, high levels of Th2-type cytokines (e.g., IL-4, IL-5, IL-6 and IL-10) tend to favor the induction of humoral immune responses. Following application of a vaccine as provided herein, a patient will support an immune response that includes Th1- and Th2-type responses. Within a preferred embodiment, in which a response is predominantly Th1-type, the level of Th1-type cytokines will increase to a greater extent than the level of Th2-type cytokines. The 20 levels of these cytokines may be readily assessed using standard assays. For a review of the families of cytokines, see Mosmann and Coffman, *Ann. Rev. Immunol.* 7:145-173, 1989.

Preferred adjuvants for use in eliciting a predominantly Th1-type response 25 include, for example, a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL), together with an aluminum salt. MPL adjuvants are available from Corixa Corporation (Seattle, WA; see US Patent Nos. 4,436,727; 4,877,611; 4,866,034 and 4,912,094). CpG-containing oligonucleotides (in which the CpG dinucleotide is unmethylated) also induce a predominantly Th1 response.

Such oligonucleotides are well known and are described, for example, in WO 96/02555 and WO 99/33488. Immunostimulatory DNA sequences are also described, for example, by Sato et al., *Science* 273:352, 1996. Another preferred adjuvant is a saponin, preferably QS21 (Aquila Biopharmaceuticals Inc., Framingham, MA), which may be used alone or in combination with other adjuvants. For example, an enhanced system involves the combination of a monophosphoryl lipid A and saponin derivative, such as the combination of QS21 and 3D-MPL as described in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol, as described in WO 96/33739. Other preferred formulations comprise an oil-in-water emulsion and tocopherol. A particularly potent adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil-in-water emulsion is described in WO 95/17210.

Other preferred adjuvants include Montanide ISA 720 (Seppic, France), SAF (Chiron, California, United States), ISCOMS (CSL), MF-59 (Chiron), the SBAS series of adjuvants (e.g., SBAS-2 or SBAS-4, available from SmithKline Beecham, Rixensart, Belgium), Detox (Corixa Corporation; Seattle, WA), RC-529 (Corixa Corporation; Seattle, WA) and aminoalkyl glucosaminide 4-phosphates (AGPs).

Any vaccine provided herein may be prepared using well known methods that result in a combination of antigen, immunostimulant and a suitable carrier or excipient. The compositions described herein may be administered as part of a sustained release formulation (i.e., a formulation such as a capsule, sponge or gel (composed of polysaccharides, for example) that effects a slow release of compound following administration). Such formulations may generally be prepared using well known technology (see, e.g., Coombes et al., *Vaccine* 14:1429-1438, 1996) and administered by, for example, oral, rectal or subcutaneous implantation, or by implantation at the desired target site. Sustained-release formulations may contain a polypeptide, polynucleotide or antibody dispersed in a carrier matrix and/or contained within a reservoir surrounded by a rate controlling membrane.

Carriers for use within such formulations are biocompatible, and may also be biodegradable; preferably the formulation provides a relatively constant level of active

component release. Such carriers include microparticles of poly(lactide-co-glycolide), as well as polyacrylate, latex, starch, cellulose and dextran. Other delayed-release carriers include supramolecular biovectors, which comprise a non-liquid hydrophilic core (e.g., a cross-linked polysaccharide or oligosaccharide) and, optionally, an external layer 5 comprising an amphiphilic compound, such as a phospholipid (see e.g., U.S. Patent No. 5,151,254 and PCT applications WO 94/20078, WO/94/23701 and WO 96/06638). The amount of active compound contained within a sustained release formulation depends upon the site of implantation, the rate and expected duration of release and the nature of the condition to be treated or prevented.

10 Any of a variety of delivery vehicles may be employed within pharmaceutical compositions and vaccines to facilitate production of an antigen-specific immune response that targets tumor cells. Delivery vehicles include antigen presenting cells (APCs), such as dendritic cells, macrophages, B cells, monocytes and other cells that may be engineered to be efficient APCs. Such cells may, but need not, be genetically 15 modified to increase the capacity for presenting the antigen, to improve activation and/or maintenance of the T cell response, to have anti-tumor effects *per se* and/or to be immunologically compatible with the receiver (*i.e.*, matched HLA haplotype). APCs may generally be isolated from any of a variety of biological fluids and organs, including tumor and peritumoral tissues, and may be autologous, allogeneic, syngeneic or xenogeneic cells.

20 Certain preferred embodiments of the present invention use dendritic cells or progenitors thereof as antigen-presenting cells. Dendritic cells are highly potent APCs (Banchereau and Steinman, *Nature* 392:245-251, 1998) and have been shown to be effective as a physiological adjuvant for eliciting prophylactic or therapeutic antitumor immunity (see Timmerman and Levy, *Ann. Rev. Med.* 50:507-529, 1999). In general, 25 dendritic cells may be identified based on their typical shape (stellate *in situ*, with marked cytoplasmic processes (dendrites) visible *in vitro*), their ability to take up, process and present antigens with high efficiency and their ability to activate naïve T cell responses. Dendritic cells may, of course, be engineered to express specific cell-surface receptors or ligands that are not commonly found on dendritic cells *in vivo* or *ex vivo*, and such

modified dendritic cells are contemplated by the present invention. As an alternative to dendritic cells, secreted vesicles antigen-loaded dendritic cells (called exosomes) may be used within a vaccine (see Zitvogel et al., *Nature Med.* 4:594-600, 1998).

Dendritic cells and progenitors may be obtained from peripheral blood, bone 5 marrow, tumor-infiltrating cells, peritumoral tissues-infiltrating cells, lymph nodes, spleen, skin, umbilical cord blood or any other suitable tissue or fluid. For example, dendritic cells may be differentiated *ex vivo* by adding a combination of cytokines such as GM-CSF, IL-4, IL-13 and/or TNF α to cultures of monocytes harvested from peripheral blood. Alternatively, CD34 positive cells harvested from peripheral blood, umbilical cord blood or 10 bone marrow may be differentiated into dendritic cells by adding to the culture medium combinations of GM-CSF, IL-3, TNF α , CD40 ligand, LPS, flt3 ligand and/or other compound(s) that induce differentiation, maturation and proliferation of dendritic cells.

Dendritic cells are conveniently categorized as "immature" and "mature" 15 cells, which allows a simple way to discriminate between two well characterized phenotypes. However, this nomenclature should not be construed to exclude all possible intermediate stages of differentiation. Immature dendritic cells are characterized as APC with a high capacity for antigen uptake and processing, which correlates with the high expression of Fc γ receptor and mannose receptor. The mature phenotype is typically characterized by a lower expression of these markers, but a high expression of cell surface 20 molecules responsible for T cell activation such as class I and class II MHC, adhesion molecules (e.g., CD54 and CD11) and costimulatory molecules (e.g., CD40, CD80, CD86 and 4-1BB).

APCs may generally be transfected with a polynucleotide encoding a 25 mammaglobin protein (or portion or other variant thereof) such that the mammaglobin polypeptide, or an immunogenic portion thereof, is expressed on the cell surface. Such transfection may take place *ex vivo*, and a composition or vaccine comprising such transfected cells may then be used for therapeutic purposes, as described herein. Alternatively, a gene delivery vehicle that targets a dendritic or other antigen presenting cell may be administered to a patient, resulting in transfection that occurs *in vivo*. *In vivo*

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and *ex vivo* transfection of dendritic cells, for example, may generally be performed using any methods known in the art, such as those described in WO 97/24447, or the gene gun approach described by Mahvi et al., *Immunology and cell Biology* 75:456-460, 1997. Antigen loading of dendritic cells may be achieved by incubating dendritic cells or 5 progenitor cells with the gammaglobin polypeptide, DNA (naked or within a plasmid vector) or RNA; or with antigen-expressing recombinant bacterium or viruses (e.g., vaccinia, fowlpox, adenovirus or lentivirus vectors). Prior to loading, the polypeptide may be covalently conjugated to an immunological partner that provides T cell help (e.g., a carrier molecule). Alternatively, a dendritic cell may be pulsed with a non-conjugated 10 immunological partner, separately or in the presence of the polypeptide.

Vaccines and pharmaceutical compositions may be presented in unit-dose or multi-dose containers, such as sealed ampoules or vials. Such containers are preferably hermetically sealed to preserve sterility of the formulation until use. In general, formulations may be stored as suspensions, solutions or emulsions in oily or aqueous 15 vehicles. Alternatively, a vaccine or pharmaceutical composition may be stored in a freeze-dried condition requiring only the addition of a sterile liquid carrier immediately prior to use.

CANCER THERAPY

20 In further aspects of the present invention, the compositions described herein may be used for immunotherapy of cancer, such as breast cancer. Within such methods, pharmaceutical compositions and vaccines are typically administered to a patient. As used herein, a "patient" refers to any warm-blooded animal, preferably a human. A patient may or may not be afflicted with cancer. Accordingly, the above pharmaceutical 25 compositions and vaccines may be used to prevent the development of a cancer or to treat a patient afflicted with a cancer. A cancer may be diagnosed using criteria generally accepted in the art, including the presence of a malignant tumor. Pharmaceutical compositions and vaccines may be administered either prior to or following surgical

removal of primary tumors and/or treatment such as administration of radiotherapy or conventional chemotherapeutic drugs.

Within certain embodiments, immunotherapy may be active immunotherapy, in which treatment relies on the *in vivo* stimulation of the endogenous host immune system to react against tumors with the administration of immune response-modifying agents (such as tumor vaccines, bacterial adjuvants and/or cytokines).

Within other embodiments, immunotherapy may be passive immunotherapy, in which treatment involves the delivery of agents with established tumor-immune reactivity (such as effector cells or antibodies) that can directly or indirectly mediate antitumor effects and does not necessarily depend on an intact host immune system. Examples of effector cells include T lymphocytes (such as CD8⁺ cytotoxic T lymphocytes and CD4⁺ T-helper tumor-infiltrating lymphocytes), killer cells (such as Natural Killer cells and lymphokine-activated killer cells), B cells and antigen-presenting cells (such as dendritic cells and macrophages) expressing a polypeptide provided herein. T cell receptors and antibody receptors specific for the polypeptides recited herein may be cloned, expressed and transferred into other vectors or effector cells for adoptive immunotherapy. The polypeptides provided herein may also be used to generate antibodies or anti-idiotypic antibodies (as described above and in U.S. Patent No. 4,918,164) for passive immunotherapy.

Effector cells may generally be obtained in sufficient quantities for adoptive immunotherapy by growth *in vitro*, as described herein. Culture conditions for expanding single antigen-specific effector cells to several billion in number with retention of antigen recognition *in vivo* are well known in the art. Such *in vitro* culture conditions typically use intermittent stimulation with antigen, often in the presence of cytokines (such as IL-2) and non-dividing feeder cells. As noted above, immunoreactive polypeptides as provided herein may be used to rapidly expand antigen-specific T cell cultures in order to generate a sufficient number of cells for immunotherapy. In particular, antigen-presenting cells, such as dendritic, macrophage or B cells, may be pulsed with immunoreactive polypeptides or transfected with one or more polynucleotides using standard techniques well known in the

art. For example, antigen-presenting cells can be transfected with a polynucleotide having a promoter appropriate for increasing expression in a recombinant virus or other expression system. Cultured effector cells for use in therapy must be able to grow and distribute widely, and to survive long term *in vivo*. Studies have shown that cultured effector cells 5 can be induced to grow *in vivo* and to survive long term in substantial numbers by repeated stimulation with antigen supplemented with IL-2 (see, for example, Cheever et al., *Immunological Reviews* 157:177, 1997).

The polypeptides provided herein may also be used to generate and/or isolate tumor-reactive T cells, which can then be administered to a patient. In one such 10 technique, antigen-specific T cell lines may be generated by *in vivo* immunization with short peptides corresponding to immunogenic portions of the disclosed polypeptides. The resulting antigen-specific CD8⁺ CTL clones may be isolated from the patient, expanded using standard tissue culture techniques and returned to the patient.

Within another embodiment, syngeneic or autologous dendritic cells may be 15 pulsed with peptides corresponding to at least an immunogenic portion of a polypeptide disclosed herein. The resulting antigen-specific dendritic cells may either be transferred into a patient or employed to stimulate T cells to provide antigen-specific T cells which may, in turn, be administered to a patient. The use of peptide-pulsed dendritic cells to generate antigen-specific T cells and the subsequent use of such antigen-specific T cells to 20 eradicate tumors in a murine model has been demonstrated by Cheever et al., *Immunological Reviews* 157:177, 1997.

Alternatively, a vector expressing a polypeptide recited herein may be introduced into antigen presenting cells taken from a patient and clonally propagated *ex vivo* for transplant back into the same patient. Transfected cells may be reintroduced into 25 the patient using any means known in the art, preferably in sterile form by intravenous, intracavitory, intraperitoneal or intratumor administration.

Routes and frequency of administration, as well as dosage, will vary from individual to individual, and may be readily established using standard techniques. In general, the pharmaceutical compositions and vaccines may be administered by injection

(e.g., intracutaneous, intramuscular, intravenous or subcutaneous), intranasally (e.g., by aspiration) or orally. Preferably, between 1 and 10 doses may be administered over a 52 week period. Preferably, 6 doses are administered, at intervals of 1 month, and booster vaccinations may be given periodically thereafter. Alternate protocols may be appropriate

5 for individual patients. A suitable dose is an amount of a compound that, when administered as described above, is capable of promoting an anti-tumor immune response, and is at least 10-50% above the basal (i.e., untreated) level. Such response can be monitored by measuring the anti-tumor antibodies in a patient or by vaccine-dependent generation of cytolytic effector cells capable of killing the patient's tumor cells *in vitro*.

10 Such vaccines should also be capable of causing an immune response that leads to an improved clinical outcome (e.g., more frequent remissions, complete or partial or longer disease-free survival) in vaccinated patients as compared to non-vaccinated patients. In general, for pharmaceutical compositions and vaccines comprising one or more polypeptides, the amount of each polypeptide present in a dose ranges from about 100 µg

15 to 5 mg per kg of host. Suitable dose sizes will vary with the size of the patient, but will typically range from about 0.1 mL to about 5 mL.

In general, an appropriate dosage and treatment regimen provides the active compound(s) in an amount sufficient to provide therapeutic and/or prophylactic benefit. Such a response can be monitored by establishing an improved clinical outcome (e.g., more

20 frequent remissions, complete or partial, or longer disease-free survival) in treated patients as compared to non-treated patients. Increases in preexisting immune responses to mammaglobin generally correlate with an improved clinical outcome. Such immune responses may be evaluated using standard proliferation, cytotoxicity or cytokine assays, which may be performed using samples obtained from a patient before and after treatment.

25

METHODS FOR DETECTING CANCER

In general, a cancer may be detected in a patient based on the presence of one or more mammaglobin epitopes or antibodies thereto in a biological sample obtained from the patient. In other words, such epitopes may be used as markers to indicate the

presence or absence of a cancer such as breast cancer. In general, such an epitope or antibody should be present at a level that is at least three fold higher in tumor tissue than in normal tissue

There are a variety of assay formats known to those of ordinary skill in the art for using a binding agent to detect polypeptide markers in a sample. See, e.g., Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. In general, the presence or absence of a cancer in a patient may be determined by (a) contacting a biological sample obtained from a patient with a binding agent; (b) detecting in the sample a level of polypeptide that binds to the binding agent; and (c) comparing the level of polypeptide with a predetermined cut-off value.

In a preferred embodiment, the assay involves the use of binding agent immobilized on a solid support to bind to and remove the polypeptide from the remainder of the sample. The bound polypeptide may then be detected using a detection reagent that contains a reporter group and specifically binds to the binding agent/polypeptide complex. Such detection reagents may comprise, for example, a binding agent that specifically binds to the polypeptide or an antibody or other agent that specifically binds to the binding agent, such as an anti-immunoglobulin, protein G, protein A or a lectin. Alternatively, a competitive assay may be utilized, in which a polypeptide is labeled with a reporter group and allowed to bind to the immobilized binding agent after incubation of the binding agent with the sample. The extent to which components of the sample inhibit the binding of the labeled polypeptide to the binding agent is indicative of the reactivity of the sample with the immobilized binding agent.

The solid support may be any material known to those of ordinary skill in the art to which the binding agent may be attached. For example, the solid support may be a test well in a microtiter plate or a nitrocellulose or other suitable membrane. Alternatively, the support may be a bead or disc, such as glass, fiberglass, latex or a plastic material such as polystyrene or polyvinylchloride. The support may also be a magnetic particle or a fiber optic sensor, such as those disclosed, for example, in U.S. Patent No. 5,359,681. The binding agent may be immobilized on the solid support using a variety

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of techniques known to those of skill in the art, which are amply described in the patent and scientific literature. In the context of the present invention, the term "immobilization" refers to both noncovalent association, such as adsorption, and covalent attachment (which may be a direct linkage between the agent and functional groups on the support or may be a linkage by way of a cross-linking agent). Immobilization by adsorption to a well in a microtiter plate or to a membrane is preferred. In such cases, adsorption may be achieved by contacting the binding agent, in a suitable buffer, with the solid support for a suitable amount of time. The contact time varies with temperature, but is typically between about 1 hour and about 1 day. In general, contacting a well of a plastic microtiter plate (such as polystyrene or polyvinylchloride) with an amount of binding agent ranging from about 10 ng to about 10 μ g, and preferably about 100 ng to about 1 μ g, is sufficient to immobilize an adequate amount of binding agent.

Covalent attachment of binding agent to a solid support may generally be achieved by first reacting the support with a bifunctional reagent that will react with both the support and a functional group, such as a hydroxyl or amino group, on the binding agent. For example, the binding agent may be covalently attached to supports having an appropriate polymer coating using benzoquinone or by condensation of an aldehyde group on the support with an amine and an active hydrogen on the binding partner (see, e.g., Pierce Immunotechnology Catalog and Handbook, 1991, at A12-A13).

In certain embodiments, the assay is a two-antibody sandwich assay. This assay may be performed by first contacting an antibody that has been immobilized on a solid support, commonly the well of a microtiter plate, with the sample, such that polypeptides within the sample are allowed to bind to the immobilized antibody. Unbound sample is then removed from the immobilized polypeptide-antibody complexes and a detection reagent (preferably a second antibody capable of binding to a different site on the polypeptide) containing a reporter group is added. The amount of detection reagent that remains bound to the solid support is then determined using a method appropriate for the specific reporter group.

More specifically, once the antibody is immobilized on the support as described above, the remaining protein binding sites on the support are typically blocked. Any suitable blocking agent known to those of ordinary skill in the art, such as bovine serum albumin or Tween 20TM (Sigma Chemical Co., St. Louis, MO). The immobilized 5 antibody is then incubated with the sample, and polypeptide is allowed to bind to the antibody. The sample may be diluted with a suitable diluent, such as phosphate-buffered saline (PBS) prior to incubation. In general, an appropriate contact time (*i.e.*, incubation time) is a period of time that is sufficient to detect the presence of polypeptide within a sample obtained from an individual with breast cancer. Preferably, the contact time is 10 sufficient to achieve a level of binding that is at least about 95% of that achieved at equilibrium between bound and unbound polypeptide. Those of ordinary skill in the art will recognize that the time necessary to achieve equilibrium may be readily determined by assaying the level of binding that occurs over a period of time. At room temperature, an incubation time of about 30 minutes is generally sufficient.

15 Unbound sample may then be removed by washing the solid support with an appropriate buffer, such as PBS containing 0.1% Tween 20TM. The second antibody, which contains a reporter group, may then be added to the solid support. Preferred reporter groups include those groups recited above.

The detection reagent is then incubated with the immobilized antibody- 20 polypeptide complex for an amount of time sufficient to detect the bound polypeptide. An appropriate amount of time may generally be determined by assaying the level of binding that occurs over a period of time. Unbound detection reagent is then removed and bound detection reagent is detected using the reporter group. The method employed for detecting the reporter group depends upon the nature of the reporter group. For radioactive groups, 25 scintillation counting or autoradiographic methods are generally appropriate. Spectroscopic methods may be used to detect dyes, luminescent groups and fluorescent groups. Biotin may be detected using avidin, coupled to a different reporter group (commonly a radioactive or fluorescent group or an enzyme). Enzyme reporter groups may

generally be detected by the addition of substrate (generally for a specific period of time), followed by spectroscopic or other analysis of the reaction products.

To determine the presence or absence of a cancer, such as breast cancer, the signal detected from the reporter group that remains bound to the solid support is generally compared to a signal that corresponds to a predetermined cut-off value. In one preferred embodiment, the cut-off value for the detection of a cancer is the average mean signal obtained when the immobilized antibody is incubated with samples from patients without the cancer. In general, a sample generating a signal that is three standard deviations above the predetermined cut-off value is considered positive for the cancer. In an alternate preferred embodiment, the cut-off value is determined using a Receiver Operator Curve, according to the method of Sackett et al., *Clinical Epidemiology: A Basic Science for Clinical Medicine*, Little Brown and Co., 1985, p. 106-7. Briefly, in this embodiment, the cut-off value may be determined from a plot of pairs of true positive rates (*i.e.*, sensitivity) and false positive rates (100%-specificity) that correspond to each possible cut-off value for the diagnostic test result. The cut-off value on the plot that is the closest to the upper left-hand corner (*i.e.*, the value that encloses the largest area) is the most accurate cut-off value, and a sample generating a signal that is higher than the cut-off value determined by this method may be considered positive. Alternatively, the cut-off value may be shifted to the left along the plot, to minimize the false positive rate, or to the right, to minimize the false negative rate. In general, a sample generating a signal that is higher than the cut-off value determined by this method is considered positive for a cancer.

For certain embodiments (*e.g.*, sandwich assays), quantitative measurements of antigen may be obtained. Within such embodiments, a standard curve may be generated. Signals obtained for antigen levels in particular samples may then be compared to the standard curve, to allow quantitation. The cut-off value within such assays may be an amount of mammaglobin indicative of the presence of breast cancer.

In a related embodiment, the assay is performed in a flow-through or strip test format, wherein the binding agent is immobilized on a membrane, such as nitrocellulose. In the flow-through test, polypeptides within the sample bind to the

immobilized binding agent as the sample passes through the membrane. A second, labeled binding agent then binds to the binding agent-polypeptide complex as a solution containing the second binding agent flows through the membrane. The detection of bound second binding agent may then be performed as described above. In the strip test format, one end 5 of the membrane to which binding agent is bound is immersed in a solution containing the sample. The sample migrates along the membrane through a region containing second binding agent and to the area of immobilized binding agent. Concentration of second binding agent at the area of immobilized antibody indicates the presence of a cancer. Typically, the concentration of second binding agent at that site generates a pattern, such as 10 a line, that can be read visually. The absence of such a pattern indicates a negative result. In general, the amount of binding agent immobilized on the membrane is selected to generate a visually discernible pattern when the biological sample contains a level of polypeptide that would be sufficient to generate a positive signal in the two-antibody sandwich assay, in the format discussed above. Preferred binding agents for use in such 15 assays are antibodies and antigen-binding fragments thereof. Preferably, the amount of antibody immobilized on the membrane ranges from about 25 ng to about 1 μ g, and more preferably from about 50 ng to about 500 ng. Such tests can typically be performed with a very small amount of biological sample.

Of course, numerous other assay protocols exist that are suitable for use 20 with the epitopes and binding agents of the present invention. The above descriptions are intended to be exemplary only. For example, it will be apparent to those of ordinary skill in the art that the above protocols may be readily modified to use polypeptides as described herein to detect antibodies that bind to such polypeptides in a biological sample. The detection of such mammaglobin epitope-specific antibodies may correlate with the 25 presence of a cancer. Other preferred assay protocols include laser scanning cytometry (a microscopic technique in which cells are stained with labeled antibody) and immunohistochemical detection. Such techniques may generally be performed according to techniques known in the art. Antibodies as provided herein may further be used to facilitate cell identification and sorting *in vitro*, permitting the selection of cells expressing

mammaglobin (or varying levels of mammaglobin). Preferably, antibodies for use in such methods are linked to a detectable marker. Suitable markers are well known in the art and include radionuclides, luminescent groups, fluorescent groups, enzymes, dyes, constant immunoglobulin domains and biotin. Within one preferred embodiment, an antibody 5 linked to a fluorescent marker, such as fluorescein, is contacted with the cells, which are then analyzed by fluorescence activated cell sorting (FACS).

In another embodiment, the above polypeptides may be used as markers for the progression of cancer. In this embodiment, assays as described above for the diagnosis 10 of a cancer may be performed over time, and the change in the level of reactive polypeptide(s) evaluated. For example, the assays may be performed every 24-72 hours for a period of 6 months to 1 year, and thereafter performed as needed. In general, a cancer is progressing in those patients in whom the level of polypeptide detected by the binding agent increases over time. In contrast, the cancer is not progressing when the level of reactive polypeptide either remains constant or decreases with time.

15 Certain *in vivo* diagnostic assays may be performed directly on a tumor. One such assay involves contacting tumor cells with a binding agent. The bound binding agent may then be detected directly or indirectly via a reporter group. Such binding agents may also be used in histological applications.

To improve sensitivity, assays as described herein may be combined with 20 assays to detect other tumor-associated antigens. It will be apparent that binding agents specific for different proteins may be combined within a single assay. The selection of tumor protein markers may be based on routine experiments to determine combinations that results in optimal sensitivity.

By alternative embodiments of the present invention, a cancer may be 25 detected in a patient based on the presence of mammaglobin polynucleotides in a biological sample obtained from the patient. In other words, such polynucleotides may be used as markers to indicate the presence or absence of a cancer such as breast cancer. In particular, polynucleotide primers and probes may be used to detect the level of mRNA encoding mammaglobin, which is indicative of the presence or absence of breast cancer. In general,

the presence of a mammaglobin polynucleotide at a level that is at least two fold, preferably at least three fold, higher than in normal tissue is indicative of breast cancer.

There are a variety of biological samples that may be used for an assay provided herein, including various body fluids and tumor samples. Preferred samples are 5 blood, and fractions thereof, such as peripheral blood, serum or plasma. In general, RNA may be isolated from blood or a fraction thereof using any standard technique.

Prior to PCR or hybridization analysis, a sample is treated by any standard technique to remove epithelial cells. It has been found, within the context of the present invention, that such treatment improves the sensitivity of the assay by up to 10 fold. One 10 method for removing epithelial cells employs Dynal's Epithelial cell enrichment beads (Dynal, Oslo, Norway), which may be used according to the manufacturer's instructions. Preferred samples for analysis are patient whole blood samples, from which epithelial cells have been removed.

Within certain embodiments, at least two oligonucleotide primers may be 15 employed in a polymerase chain reaction (PCR) based assay to amplify a portion of a mammaglobin cDNA derived from a biological sample, wherein at least one of the oligonucleotide primers is specific for (i.e., hybridizes to) a polynucleotide encoding mammaglobin. The amplified cDNA is then separated and detected using techniques well known in the art, such as gel electrophoresis and autoradiography. Similarly, 20 oligonucleotide probes that specifically hybridize to a mammaglobin polynucleotide may be used in a hybridization assay to detect mammaglobin expression in a biological sample.

To permit hybridization under assay conditions, oligonucleotide primers and probes should comprise an oligonucleotide sequence that has at least about 60%, preferably at least about 75% and more preferably at least about 90%, identity to a portion of a 25 mammaglobin polynucleotide that is at least 10 nucleotides, and preferably at least 20 nucleotides, in length. Oligonucleotide primers and/or probes which may be usefully employed in the diagnostic methods described herein preferably are at least 10-40 nucleotides in length. Techniques for both PCR based assays and hybridization assays are

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well known in the art (see, for example, Mullis et al., Cold Spring Harbor Symp. Quant. Biol. 51:263, 1987; Erlich ed., PCR Technology, Stockton Press, NY, 1989).

One preferred assay employs RT-PCR, in which PCR is applied in conjunction with reverse transcription. Typically, RNA is extracted from a sample tissue 5 and is reverse transcribed to produce cDNA molecules. PCR amplification using at least one specific primer generates a cDNA molecule, which may be separated and visualized using, for example, gel electrophoresis. Amplification may be performed on samples obtained from biological samples taken from a test patient and an individual who is not afflicted with a cancer. The amplification reaction may be performed on several dilutions 10 of cDNA spanning two orders of magnitude. A two-fold or greater increase in expression in several dilutions of the test patient sample as compared to the same dilutions of the non-cancerous sample is typically considered positive.

Yet another amplification technique that may be used within such assays is real-time PCR (see Gibson et al., Genome Research 6:995-1001, 1996; Heid et al., Genome 15 Research 6:986-994, 1996). Real-time PCR is a technique that evaluates the level of PCR product accumulation during amplification, permitting quantitative evaluation of mRNA levels. Briefly, mRNA is initially extracted from cells of interest using standard techniques. Real-time PCR may then be performed, for example, using a Perkin Elmer/Applied Biosystems (Foster City, CA) 7700 Prism instrument. Matching primers 20 and fluorescent probes may be designed for mammaglobin using, for example, the primer express program provided by Perkin Elmer/Applied Biosystems (Foster City, CA). Optimal concentrations of primers and probes may be initially determined by those of ordinary skill in the art, and control (e.g., β -actin) primers and probes may be obtained commercially from, for example, Perkin Elmer/Applied Biosystems (Foster City, CA). To 25 quantitate the amount of mammaglobin RNA in a sample, a standard curve may be generated alongside using a plasmid containing a mammaglobin gene. Standard dilutions ranging from $10-10^6$ copies of the gene of interest are generally sufficient. In addition, a standard curve may be generated for the control sequence, to permit standardization of initial RNA content of a tissue sample to the amount of control for comparison purposes.

Certain in vivo diagnostic assays may be performed directly on a tumor. One such assay involves contacting tumor cells with a polynucleotide probe. Bound probe may be detected directly or indirectly using a reporter group.

As noted above, to improve sensitivity, multiple breast tumor protein 5 markers may be assayed within a given sample. For example, a polynucleotide probe or primer as described herein may be used concurrently with a probe or primer designed to detect a different marker. The selection of breast tumor markers may be based on routine experiments to determine combinations that results in optimal sensitivity.

10 DIAGNOSTIC KITS

The present invention further provides kits for use within any of the above diagnostic methods. Such kits typically comprise two or more components necessary for performing a diagnostic assay. Components may be compounds, reagents, containers and/or equipment. For example, one container within a kit may contain a monoclonal 15 antibody or fragment thereof that specifically binds to a mammaglobin epitope. Such antibodies or fragments may be provided attached to a support material, as described above. One or more additional containers may enclose elements, such as reagents or buffers, to be used in the assay. Such kits may also, or alternatively, contain a detection reagent as described above that contains a reporter group suitable for direct or indirect 20 detection of antibody binding.

Preferred kits are those designed for use within sandwich assays. Such kits comprise two or more components for use within such assays. For example, such a kit may comprise standards based on recombinant mammaglobin for use in preparing a standard curve. Such a kit may comprise one or both antibodies for use within the assay (i.e., the 25 capture antibody and/or signal antibody), with or without additional reagents for use in detecting mammaglobin binding.

Kits designed to detect the level of mRNA encoding mammaglobin in a biological sample may comprise at least one oligonucleotide probe or primer, as described above. Such an oligonucleotide may be used, for example, within a PCR or hybridization

assay. Additional components that may be present within such kits include a second oligonucleotide and/or a diagnostic reagent or container to facilitate the detection of a mammaglobin polynucleotide.

5 The following Examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1Identification of Mammaglobin Epitopes and Preparation of Antibodies

5

This Example illustrates the preparation of anti-mammaglobin antibodies and epitope mapping.

Rabbits and mice were immunized with full-length human mammaglobin protein. Mouse monoclonal antibodies were isolated with standard hybridoma technology.

10 Rabbit monoclonal antibodies were isolated with selected lymphocyte antibody method (SLAM) technology. In addition to these antibodies, a purified polyclonal antibody directed against the C-terminus of mammaglobin was also developed following immunization of rabbits with a C-terminal peptide.

15 Figure 1A illustrates the monoclonal antibodies that were developed for mammaglobin. For the rabbit monoclonal antibodies the Ig variable regions were sequenced. The sequence for the variable regions of each rabbit antimammaglobin monoclonal antibody is shown in Figures 1B-1C.

20 In order to better define the epitope binding region of each monoclonal antibody a series of peptides was generated that spans the entire mammaglobin protein sequence. The amino acid sequence for mammaglobin is shown in Figure 2, and the corresponding peptides are indicated. In addition to the peptides, a short recombinant form of mammaglobin was generated by cleavage with protease. 96 well microtiter plates (Costar) were coated with either peptide or recombinant antigen at 200 ng/well. Coating was overnight at 4°C. Plates were then aspirated and blocked with phosphate buffered saline containing 1% (w/v) BSA for 2 hours at room temperature, and then washed in PBS containing 0.1% Tween 20 (PBST). Purified rabbit antibodies at different dilutions (1000 to 7.8 ng/ml) in PBST was added to the wells and incubated for 30 minutes at room temperature. This was followed by washing 6 times with PBST and then incubating with Protein-A HRP conjugated at a 1/20000 dilution for a further 30 minutes. Plates were 25 washed 6 times in PBST and then incubated with Tetramethylbenzidine (TMB) substrate 30

for a further 15 minutes. The reaction was stopped by the addition of 1 N sulfuric acid and plates were read at 450 nm using an ELISA plate reader.

ELISA with the mouse monoclonal antibodies was performed with supernatants from tissue culture run neat in the assay.

5 A summary of the data is shown in Figure 3A. Shaded cells are considered positive for the antibody. The reactivity of three different epitopes is shown in Figures 3B, 3C and 3D, where 2D3 reacts with pro5 and the N-terminal recombinant and 29C11 reacts weakly with pro2. The epitope binding sites of the antimammaglobin antibodies are summarized in Figure 1A.

10 Subsequent to epitope mapping, the antibodies were tested by FACS analysis on a cell line that expresses mammaglobin, MB415 breast carcinoma cells. In order to ensure specificity of antibody binding, MCF-7 cells that do not express mammaglobin were also tested by FACS analysis under identical conditions. Cells were fixed with 4% formaldehyde for 20 min before being washed 2 times. Cells were then 15 permeabilized for 10 minutes with PBS containing 0.1% saponin. 0.5 µg of anti-mammaglobin monoclonal antibody was added and cells were incubated at room temperature for 30 minutes before being washed 2 times and incubated with a FITC-labeled goat anti-rabbit or mouse secondary antibody for 20 minutes. After being washed 2 times, cells were analyzed with an Excalibur fluorescent activated cell sorter. The results are 20 illustrated in Figure 4A.

Western blot analysis was also used to characterize anti-mammaglobin monoclonal specificity (Figure 5). SDS-PAGE was performed on 1) media in which MDA-MB-415 cells were grown, 2) MDA-MB-415 cell lysate and 3) bacterially expressed recombinant mammaglobin. Protein was transferred to nitrocellulose and then Western 25 blotted for the antimammaglobin monoclonal antibodies at an antibody concentration of 1 µg/ml. Protein was detected using horse radish peroxidase (HRP) conjugated to either a goat anti-mouse monoclonal antibody or to protein A-Sepharose. The purified anti-mammaglobin polyclonal antibody recognized bacterial expressed recombinant mammaglobin, as well as mammaglobin expressed in and secreted from MDA-MB-415

breast carcinoma cells. All mouse and rabbit monoclonal antibodies recognized recombinant bacterial expressed mammaglobin. With the exception of 197-1H11, all of the mouse monoclonal antibodies recognized mammaglobin secreted into the cell media or expressed within the cytoplasm. The rabbit monoclonal antibodies 14A12, 6B12 and 2D3 5 recognized recombinant bacterial expressed mammaglobin, as well as mammaglobin expressed in the cytoplasm and secreted into the media. Although unable to recognize mammaglobin secreted into the media, rabbit monoclonal antibody 6A1 was able to recognize bacterial expressed mammaglobin and mammaglobin expressed in the cytoplasm of MB415 cells. The inability of monoclonal antibodies 197-1H11 and 6A1 to associate 10 with specific forms of mammaglobin likely reflect differential posttranslational modifications such as glycosylation and/or relative affinity of the antibody to mammaglobin.

In order to determine which tissues express mammaglobin, immunohistochemistry (IHC) analysis was performed on a diverse range of tissue sections. Tissue samples were fixed in formalin solution for 24 hours and embedded in paraffin 15 before being sliced into 10 micron sections. Tissue sections were permeabilized and incubated with anti-mammaglobin antibody for 1 hour. HRP-labeled anti-mouse or anti-rabbit antibody was used to visualize mammaglobin immunoreactivity. Figure 6 summarizes the tissue-specific distribution of mammaglobin protein. Mammaglobin was highly expressed in breast tissue but not found in other tissues tested including adrenal, 20 cervix, colon, duodenum, gall bladder, ileum, kidney, ovary, pancreas, parotid gland, prostate, skeletal muscle, spleen, and testis.

Example 2

Sandwich Immunoassays for Mammaglobin

25 This Example illustrates the use of antibodies provided herein for detection of mammaglobin in serum.

Monoclonal antibodies and the rabbit polyclonal antibody 967 directed to the C-terminal 16 amino acid peptide of mammaglobin were evaluated in sandwich

ELISAs for their ability to detect mammaglobin in lysates of MB415 cells, cell supernatants of MB415 cells and also in the serum of breast cancer patients. Antibodies were paired based on their ability to detect different epitopes. The following describes some of the sandwich combinations tested. In all assays a standard curve was constructed
5 by spiking recombinant mammaglobin into male serum.

Mouse/Rabbit Antibody Sandwiches

Assays were designed to capture the mouse monoclonal antibody using a solid phase of goat anti-mouse IgG as part of the sandwich. 96 well plates (Costar 10 Corning) were coated overnight at 4°C with 200ng/well of goat anti mouse IgG (Rockland antibodies, Rockland, ME). Plates were washed in Phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST) then blocked with 1% BSA in phosphate buffered saline(PBS) for 2 hours. Mouse monoclonal supernatants were then added (50ul) at 1:10 dilution in PBS and the plates incubated for a further hour at room temperature. Plates 15 were washed six times in PBS Tween 20 (PBST) then blocked with 1% normal mouse serum and 1% normal human serum for 1 hour then washed again. Samples and standards were applied to the wells and the plate was incubated for 1 hour at room temperature. The plate was then washed six times in PBS containing 0.05% Tween. Biotinylated 31A5 or 2D3 were used as the conjugate at 1ug/ml in PBS and 1% Normal mouse serum. These 20 were incubated for 1 hour at room temperature then washed six times in PBST. 50μl of a 1:10000 dilution of streptavidin HRP in PBS containing 1% normal mouse serum was added and the plate incubated at room temperature for 30 minutes at which time the plate was again washed six times. TMB (tetramethyl benzidine) substrate (Kirkegaard and Perry) was then added to the well and incubated for a further 15 minutes. The reaction was 25 stopped with 1N H₂SO₄ (100μl) and the signal generated read at 450nm. A standard curve relating pg mammaglobin in the assay was constructed using recombinant mammaglobin spiked into normal male serum and samples run in the test were quantitated using this standard curve.

Rabbit/Rabbit antibody sandwiches

Two assays were performed. The first utilized affinity purified rabbit anti 967 peptide (C-terminal 16 amino acid peptide) as the solid phase and 2D3 rabbit monoclonal biotinylated as the signal antibody. The second used 2D3 as the solid phase 5 antibody and 31A5-biotinylated as the signal antibody. In the first assay the affinity purified polyclonal antibody was coated overnight at 4°C on a 96 well plate (200ng/well) in 50 mM carbonate/bicarbonate buffer pH9.5. Plates were washed in PBST then blocked for 2 hours at room temperature with 1% BSA in PBS. Serum (50µl) was then added to the plate and incubated for 1 hour at room temperature. Plates were then washed six times 10 with Phosphate buffered saline containing 0.05% Tween 20. Biotinylated 2D3 monoclonal antibody (2ug/ml) in PBS containing 1% normal rabbit serum was then added and the plate further incubated at room temperature for 1 hour. After again washing six times, 50µl of a 1:10000 dilution of streptavidin HRP in PBS containing 1% normal rabbit serum was added and the plate incubated at room temperature for 30 minutes at which time the plate 15 was again washed six times. Development of signal with TMB substrate was as described above.

In the second assay 2D3 was coated on 96 well plates at 200ng/well (Costar/Corning, Cambridge MA) overnight at 4°C washed in PBST then blocked for two hours at room temperature. Serum (50µl) was then added to the plate and incubated for 1 20 hour at room temperature. Plates were then washed six times with Phosphate buffered saline containing 0.05% Tween 20. Biotinylated 31A5 monoclonal antibody (0.5µg/ml) in PBS containing 1% normal rabbit serum was then added and the plate further incubated at room temperature for 1 hour. After again washing six times the streptavidin-HRP and TMB incubations were performed as described above.

25 Figures 7A-7C are examples of the sandwiching of the mouse monoclonal antibodies with 31A5, 6B12 or 2D3 biotinylated and the ability to detect mammaglobin in MB415 lysates as well as supernatants. In Figure 8 is shown the linear portion of the standard curve for the polyclonal anti-967 serum in combination with the monoclonal 2D3 biotinylated. This curve was used to quantitate mammaglobin serum samples of 7 patients

with metastatic breast cancer. Of these 5/7 were positive for mammaglobin with serum levels in the 1-10ng/ml range. In the same experiment 9/11 normal samples were negative and below the cut-off. Mammaglobin and mammaglobin RNA were detectable in these same breast cancer patient samples using a sandwich of 2D3 and 29C11 as shown in Figure 5 9. For all experiments, mammaglobin levels were obtained using a standard curve, and negative and positive controls were as expected.

Example 3

Identification of Anti-oligosaccharide Antibodies Specific for Mammaglobin

10

This Example illustrates the preparation of antibodies that specifically bind mammaglobin in a glycosylation-sensitive manner, including antibodies to differentially glycosylated sites of mammaglobin expressed in breast cancer cells.

An antibody library (Glycotech Corp., Rockville MD) that encompasses 20-15 30 antibodies to different glycosylation combinations is screened using native mammaglobin from breast carcinoma cell lines via conventional ELISA and blotting techniques. Native mammaglobin is purified from MDA-MB-415 breast carcinoma cells using standard biochemical purification procedures. Both rabbits and mice are immunized with native mammaglobin. SLAM technology is used to develop rabbit monoclonal 20 antibodies that bind to native mammaglobin but not to mammaglobin that has been stripped of oligosaccharides using deglycosylation enzymes. Identical approaches are used to screen hybridoma supernatants that are generated from mice immunized with native mammaglobin.

The glycosylation epitopes for the antibodies generated in rabbits and mice 25 are mapped using a carbohydrate library (Glycotech Corp.). The carbohydrate library consists of a diverse array of oligosaccharide combinations permits the definition of carbohydrate epitopes to the antibodies. Upon identification, isolation and characterization of anti-oligosaccharide antibodies specific for mammaglobin, a sandwich ELISA assay is performed in which mammaglobin is captured from the sera of breast cancer patients with

an anti-mammaglobin polyclonal or monoclonal antibody (which binds an epitope that is the 16 C-terminal amino acids of mammaglobin) and the anti-carbohydrate antibody is used for detection. The resulting ELISA assay provides sensitive and accurate diagnosis of breast cancer.

5

Example 4

Identification of Human CD4 T cell Epitopes for Mammaglobin

This Example illustrates the generation of CD4 T cells that recognize
10 mammaglobin.

CD4 T cell responses were generated from PBMC of normal donors using dendritic cells (DC) pulsed with overlapping 20-mer peptides spanning the entire mammaglobin protein sequence. CD4⁺ T cells were stimulated 3-4 times with DC pulsed with a mixture of overlapping peptides (10 µg/mL each) in Iscove's modified Dulbecco's
15 Medium (IMDM) containing IL-6 and IL-12 in the primary stimulation, and 0.5 ng/mL IL-2 and 5 ng/mL IL-7 in all other stimulations. The peptides are shown in Figure 10. These lines were subsequently assayed for reactivity with the priming peptides or recombinant *E. coli*-derived mammaglobin. As shown in Figures 11A and 11B, a number of CD4 T cell lines demonstrated reactivity with the priming peptides as well as mammaglobin protein.
20 The peptide specific reactivity of these lines was demonstrated in lines primed with peptides 3A (aa 21-40), 5A (aa 41-60) and 7A 9aa 61-80 of the mammaglobin protein sequence. The dominant reactivity of these lines appeared with peptide 5A (EYKELLQE-FIDDNATTNAID; SEQ ID NO: 4), corresponding to amino acids 41-60 of the mammaglobin sequence. These results indicate that peptide 5A represents an immunogenic
25 CD4 epitope of mammaglobin. Figure 18 shows the results of an assay for CD4⁺ proliferation.

Example 5

Identification of Human CD8 T cell Epitopes for Mammaglobin

This Example illustrates the generation of CD8 T cells that recognize
5 mammaglobin.

HLA A2Kb mice were immunized with 9-mer peptides predicted to bind HLA A2 (shown in Figure 12). Immunizations were performed subcutaneously in the footpad, using 100 µg of peptide together with 140 µg of hepatitis B virus core peptide (a Th peptide) in Freund's incomplete adjuvant. Three weeks post immunization, spleen cells
10 were removed and cultured *in vitro* with peptide-pulsed APC to elicit CTL lines. CTL lines were subsequently evaluated for recognition of peptide-pulsed mammaglobin-transduced target cells in a standard chromium release assay. CTL lines recognizing peptide pulsed targets were then tested on targets transduced and stably expressing the mammaglobin protein. CTL lines from mice immunized with the 9-mer peptide
15 KLLMVLMLA (mgb 1; SEQ ID NO: 5) corresponding to amino acids 2-10 of mammaglobin were shown to recognize both peptide-pulsed and mammaglobin transduced targets (Figures 13A-13C and 14A-14C). These data demonstrate that the 9-mer peptide KLLMVLMLA (SEQ ID NO: 5) is a naturally processed CTL epitope of mammaglobin, and is restricted by HLA A2.

20

Example 6

Detection of Mammaglobin RNA in Patient Blood Samples

This Example illustrates the use of PCR to detect mammaglobin expression
25 in blood for the purpose of diagnosing breast cancer.

RNA extraction: RNA was extracted from frozen tumors and normal tissues and cell lines (MB415) as follows. Tissue samples were homogenized in Trizol reagent (Gibco, BRL) at 1ml/50-100mg of tissue using a homogenizer (Polytron) and cells mixed with Trizol reagent at 1ml 5-10x10⁶ cells. The homogenized samples were then incubated
30 at room temperature for 5 minutes followed by the addition of 0.2ml of chloroform per 1ml

of Trizol reagent. Sample tubes were capped and vigorously shaken for 15 seconds followed by a further incubation at room temperature for 2-3 minutes. Samples were centrifuged at 12,000g for 15 minutes at 2-8°C, and the upper aqueous phase was removed. The RNA preparation was transferred to a new tube and precipitated by addition of 0.5ml 5 isopropyl alcohol per 1ml Trizol reagent used in the homogenation step. Samples were incubated at room temperature for 10 minutes, and then centrifuge for 10 minutes, 12,000g at 2-8°C. The supernatant was removed from the gel like pellet and the pellet was washed once with 75% ethanol (1 ml/1ml of Trizol). The sample was mixed and then centrifuged at 7,500g for 5 minutes at 2-8°C. Supernatant was removed and the RNA pellet was briefly 10 dried at room temperature and dissolved in RNase free water.

Isolated RNA was treated with DNase to remove any DNA contamination. The RNA (50µg) in 75µl nuclease free water and first strand buffer (Gibco BRL) was incubated with DNaseI (Ambion) in the presence of RNase inhibitor RNasin (Promega) at 37°C for 30 minutes. The reaction mix was then precipitated with phenol/chloroform and 15 centrifuged for 5 minutes in an eppendorf centrifuge maximum speed. The top layer was transferred to new tube, to which 20µl 3M sodium acetate and 440µl of 100% cold ethanol was added. The mixture was vortexed and spun again for 5 minutes. Supernatant was discarded and the pellet was washed with 75% cold ethanol and centrifuged. The RNA pellet was resuspended in RNase free water at 1-2µg/ml.

20 RNA was extracted from whole blood using Dynal's Epithelial cell enrichment beads and Dynal's mRNA Direct kit (Dynal, Oslo, Norway) according to the manufacturer's instructions. RNA extracted via the Dynal extraction kit was immediately resuspended in 20ml of Reverse transcription mix shown below and reverse transcribed.

Reverse Transcription: cDNA for use in real time PCR tissue panels was 25 prepared as follows. 25µg of RNA was incubated with 25µl Oligo dT (Boehringer Mannheim) (100ng/ml) at 70°C for 10 minutes, and then with 125µl of diluted reverse transcriptase buffer (Gibco, BRL containing 0.5mM dNTP's 1000 units RNasin, 0.02mM dithiothreitol and Superscript II (Gibco BRL) at 42°C for 1 hour. The reaction mix was then cooled to 4°C for use in real-time PCR or frozen. The reaction mix for the epithelial

extracted material was 20 μ l of Superscript RT mix (4 μ l of 5x buffer, 2 μ l of 0.1M DTT, 1 μ l 10mM dNTP mix, 1 μ l (200 units) of superscript II and 12 μ l of RNase free water. The mix was then incubated at 50°C for 5 minutes followed by 42°C for 50 minutes then inactivated at 70°C for 15 minutes.

5 Real Time PCR: Real time PCR analysis was performed on the Perkin
Elmer/Applied Biosystems 7700 Prism instrument. Matching primers and fluorescent
probes were designed for each of the genes of interest according to the primer express
program provided by Perkin Elmer/Applied Biosystems (Foster City, CA). Primers and
probes so produced can be used in the universal thermal cycling program in Real time
10 PCR. Initially the primers and probes were titrated to determine the optimal concentrations
using a checkerboard approach. A pool of cDNA from target tumors was used in this
optimization process. These reagents were then used in Real Time PCR at their optimal
concentrations. The reaction was performed in 25 μ l volumes. In all cases the final probe
concentration was 155nM. dATP, dCTP and dGTP were at 0.2mM and dUTP at 0.4mM.
15 AmpliTaq gold and Amperase UNG (Perkin Elmer/Applied Biosystems, Foster City CA)
were used at 0.625 units and 0.25 units per reaction. MgCl₂ was at a final concentration of
5mM. Trace amounts of glycerol, gelatin and Tween 20 (Sigma Chem Co, St Louis, MO)
were added to stabilize the reaction. Each reaction contained 2 μ l of template. B-actin
20 primers and probes were obtained from Perkin Elmer/Applied Biosystems (Foster City,
CA) and used in a similar manner to quantitate the presence of B-actin in the samples. The
forward primer was at 900nM, reverse primer at 300nM.

In order to quantitate the amount of specific RNA in the sample a standard curve was generated alongside using the plasmid containing the gene of interest. Standard curves were generated using the Ct values determined in the real-time PCR which are related to the initial cDNA concentration used in the assay. Standard dilutions ranging from 10-10⁶ copies of the gene of interest were used for this purpose. In addition, a standard curve was generated for β-actin ranging from 200fg-2000pg. This enabled

standardization of initial RNA content of a tissue sample to the amount of β -actin for comparison purposes.

The primers and probes used were as shown in Table 1.

Table 1Mammaglobin Primers and Probes

	Mammaglobin	SEQ ID NO:
Forward Primer	TGCCATAGATGAATTGAAGGAATG	8
Reverse Primer	TGTCATATATTAATTGCATAAACACCTCA	9
Probe	TCTTAACCAAACGGATGAAACTCTGAGCAATG	10

5

The mammaglobin gene sequence contains three exons. Exon one spans bases 992 through 1110, exon two from 1713 through 1900, and exon three from 3789 through 3974. The start Met is at base 1056 and the stop codon is at base 3725. The primers and probes used for the quantitative real time PCR are located in exon 2; however, 10 the reverse primer is divided between exon 2 and exon 3. The primer placement does not exclude amplification of genomic DNA. All tissue samples were DNase treated with Ambion DNase I. These samples were tested for the presence of contaminating DNA prior to use. RNA extracted from whole blood using Dynal's Epithelial cell enrichment beads and Dynal's mRNA Direct kit were not DNase treated, but this is a highly specific isolation 15 method for RNA only.

Figure 15 shows the tissue distribution for mammaglobin showing the high degree of specificity for breast tissue. The skin sample shown to be positive was from a breast reduction. Figure 16 shows the copies of mammaglobin message detectable in the breast cancer cell line MB415 as a function of the amount of cells indicating that one cell 20 has \sim 10000 copies. Figure 17 shows the detection of mammaglobin in epithelial cells isolated, using the Dynal isolation method, from the peripheral blood of patients with metastatic breast cancer compared to similar isolates from normal blood samples. Thirty three metastatic and 11 normal samples were tested. The data indicate that mammaglobin can be detected in the blood of individuals with metastatic breast cancer, and that such 25 detection may be used to diagnose the disease.

Example 7

Demonstration of tumor recognition by mammaglobin-specific CD4 T Cells

5 CD4⁺ T cells specific for mammaglobin were generated as described above. Briefly, CD4⁺ T cells were primed in vitro using dendritic cells and overlapping peptides spanning the entire mammaglobin protein sequence. Lines with peptide reactivity were screened for recognition of recombinant E. coli-derived mammaglobin protein. T cell lines demonstrating activity to recombinant mammaglobin were selected for further
10 characterization.

To determine whether these CD4⁺ T cell lines were able to recognize native mammaglobin secreted from tumor cells or tumor cells expressing mammaglobin, the lines were tested for reactivity with the following:

- recombinant E. coli derived mammaglobin
- 15 - native mammaglobin purified from MB415 (mammaglobin+) breast tumor cells
- deglycosylated native mammaglobin
- denatured native mammaglobin
- denatured, deglycosylated native mammaglobin
- 20 - MB415 tumor cell lysate (mammaglobin+ breast tumor cells)
- SKOV3 tumor cell lysate (mammaglobin- ovarian tumor cells)

The results, shown in Table 2, demonstrate that lines #8 and #19 recognize native mammaglobin in the deglycosylated form. In addition, one line (line #13) recognizes native mammaglobin, and MB415 lysate as well as the deglycosylated forms of native mammaglobin demonstrating that this line recognizes mammaglobin expressed by tumor cells. In a subsequent assay (Figure 19), 2 additional lines (#14 and #27) were also shown to recognize native mammaglobin and MB415 tumor cell lysate.
25

TABLE 2

Reactivity with Mammoglobin	Cell Line		
	Line 8*	Line 13	Line 19
Medium	1	1	1
Recombinant E. coli- Derived	145	375	300
Native	2	50	1
Denatured Native	1	25	1
Deglycosylated Native	135	375	35
Denatured, Deglycosylated Native	2	430	1
MB415	1	40	1
SKOV3	2	0	1

*Values represent the stimulation index relative to the medium control.

5

The CD4 peptide epitope recognized by each of these lines is listed below in Table 3.

Table 3

Line #	Peptide(s) Recognized
13	3A, 7A
8	7A
19	5A
14	3A, 5A, 7A
27	3A

10

In summary, these results demonstrate that mammoglobin-specific CD4 T cells can be generated in vitro by priming with peptides. Furthermore, some of these T cell

lines demonstrate significant reactivity with mammaglobin-expressing tumor cells and/or mammaglobin purified from tumor cells.

Example 8

5 Expression of Recombinant Ra12 (s) MammFL, a fusion protein consisting of full length
human Mammaglobin with short Ra12

A Ra12 (s) MammFL construct was engineered, consisting of short Ra12 linked to full length human mammaglobin. The fusion protein was produced in *E. coli*, and
10 the recombinant protein is suitable for breast cancer therapy and diagnostics. The DNA sequence is shown in SEQ ID NO:46 and the protein sequence is shown in SEQ ID NO:47.

This example provides recombinant, Ra12(s)MammFL, which is an N-terminal Ra12 (short) fusion to full length human mammaglobin gene. The recombinant Ra12(s)MammFL is 132aa protein.

15 Recombinant versions of the mammaglobin gene have been made, but a full length version, including the N-terminal signal peptide, has not been made in an *E. coli* expression vector. Using hMamm in pcDNA 3.1, a midprep (Wizard) was performed to obtain purified vector containing hMamm for use as template in PCR. Primers FLMamm1 (SEQ ID NO:48) and FLMamm2 (SEQ ID NO:49) were used to PCR amplify the full
20 length gene, adding 5' Hind III and 3' Xho I sites for easy subcloning into pCRX1 vector. This subcloning strategy put the full length mammaglobin gene in frame at the 3' end of the short form of Ra12. Vector and insert were digested with Hind III and Xho I. Vector was additionally treated with CIAP to prevent self-ligation. Ligation was done with pCRX1 and FLMamm at room temperature. Ligation mix was used to transform NovaBlue
25 *E. coli* host. Transformed *E. coli* was plated and grown overnight. Ten colonies were screened for insert by digestion with Hind III and Xho I. Positive clones were sequenced using T7 promoter and terminator primers. Sequencing confirmed pCRX1 Ra12(s)MammFL #1 (#61474). This clone was transformed into *E. coli* BLR (DE3) and HMS 174 (DE3) host strains for protein expression.

Cloning Primers:

Sense Primer - FLMamm1 #7769 FL Mamm 5' primer, Hind III site
5'-gcgaagttATGAAGTTGCTGATGGTCCTCATGC-3' (SEQ ID NO:48)

5 full length 34bp 50% 66C Tm
complement 25bp 48% 58C Tm

Antisense Primer-FLMamm2 #7770 FL Mamm 3' primer, Xhol site

5'-cggctcgagTTAAAATAAATCACAAAGACTGCTGTC-3'
10 (SEQ ID NO:49)

full length 36bp 42% 63C Tm
complement 27bp 30% 52C Tm

The Ra12(s)MammFL has the following characteristics - molecular weight
15 of 14638.81 Daltons; 132 amino acids; isoelectric point of 4.936, and charge at pH 7.0 of
-8.033.

Protein Expression:

A standard "mini" expression screen was performed to determine the
20 optimal induction conditions. BLR (DE3) cells grew slowly after inoculation, HMS 174
(DE3) grew faster, but both were induced achieving peak cell density only 1 hr after
induction (though expression levels are highest 3hr post-induction). Coomassie stained
SDS-PAGE showed a specifically induced band at about 14kD (Figure 21). HMS 174
25 (DE3) strain grew the best, with optimal expression of Ra12(s)MammFL in TBS media at
37°C induced for 3hr.

From the foregoing it will be appreciated that, although specific
embodiments of the invention have been described herein for purposes of illustration,

various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

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